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# HETEROTROPHIC GROWTH OF MICROALGAE *CHLORELLA PROTOTHECOIDES* FOR LIPID PRODUCTION AND THEIR EXTRACTION USING SUPERCRITICAL $CO_2$

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# Abstract

This thesis deals with a project on the extraction of oil from microalgae using supercritical $CO_2$, with the purpose of producing biodiesel from the extracted oil.

The strain of microalgae used in this study was *Chlorella protothecoides*, which was grown under heterotrophic conditions, a culture technique which has proved to be very effective, with the aim of increase the amount of oil produced by microalgae. The influence of different culture volumes and media, light conditions, nutrient intake and pH conditions were tested. The best result was obtained with a 3.5-l aquarium, reaching a biomass concentration of 44.5 g/l in 185 h of culture. Further improvements could be achieved through a better control of temperature and air flow rate.

On the other hand, supercritical extraction was performed on biomass samples with an average oil content of 35%wt., obtained from a previous study. Factors affecting fluid dynamics and external mass transfer, like the effect of bed geometry and $CO_2$ flow rate, and operating conditions affecting solvation capacity of $CO_2$, like the effect of pressure and temperature, were tested. The operative conditions were 150 and 300 bar, 35 and 70°C, 1, 3 and 5 g $CO_2$/min in various combinations, in order to study their influence on the extraction rate and time.

A good compromise between high extraction yields and short extraction times was achieved with 3 g $CO_2$/min flow rate at 300 bar and 35°C. Further optimization of pressure and temperature conditions using this flow rate is expected to improve the process.

# Riassunto

Questa tesi si è occupata di un progetto sull'estrazione di olio da microalghe con CO2 supercritica, con lo scopo di produrre biodiesel dall'olio estratto.
Gli obiettivi di questo progetto erano due; innanzitutto, sviluppare un sistema economico, rispetto ad un bioreattore automatico, al fine di ottimizzare le condizioni di crescita di un promettente ceppo eterotrofo di microalghe, *Chlorella protothecoides* (UTEX 249). In secondo luogo, sono state eseguite varie estrazioni con $CO_2$ supercritica al fine di migliorare i risultati ottenuti nello studio di Viguera *et al.* (2012), utilizzando la stessa biomassa, ma modificando il tipo di estrattore, la sua geometria e le condizioni operative.
Negli ultimi anni, l'aumento del consumo e dei prezzi delle energie convenzionali, l'esaurimento dei combustibili fossili, il riscaldamento globale e le emissioni di gas serra hanno causato serie preoccupazioni riguardo la sicurezza energetica e il degrado ambientale. Pertanto, l'energia ricavata dalle biomasse (rinnovabile e non inquinante) sta ricevendo sempre più attenzione da parte sia della comunità accademica sia delle industrie. Tuttavia, l'uso di grandi quantità di piante commestibili per la produzione di biocarburanti ha sollevato molte critiche per le varie questioni etiche in gioco e, per questo motivo, le microalghe hanno attratto l'attenzione come una possibile soluzione a questi problemi imminenti.
Le microalghe hanno diversi vantaggi rispetto alle biomasse oleaginose convenzionali, quali: strutture più semplici, ma alta efficienza foto sintetica e possono essere prodotte tutto l'anno; sono molto idonee per produrre biodiesel grazie al loro elevato contenuto lipidico (solitamente dal 20% al 50% su base secca), e possono essere coltivate in acqua dolce, laghi salati, oceani, terre marginali, deserti, ecc. e non c'è competizione con i terreni che potrebbero essere utilizzati per colture alimentari; rimuovono efficacemente i nutrienti come l'azoto e il fosforo, e metalli pesanti dalle acque reflue, sequestrano una grande quantità di atomi di carbonio attraverso la fotosintesi, per cui l'utilizzo di $CO_2$ delle centrali termoelettriche può ridurre di molto le emissioni di gas a effetto serra, responsabili del riscaldamento globale.
Una delle specie più promettenti di microalghe è il ceppo *Chlorella protothecoides*, che è stato scelto per le finalità del presente lavoro. Infatti, in letteratura è stato riportato un contenuto lipidico massimo di 57,8%. Questo ceppo è in grado di crescere sia in condizioni autotrofe che eterotrofe e, in particolare, la crescita eterotrofa è considerata

come il modo più pratico e promettente per aumentare la produttività di lipidi da parte di questi microrganismi. Inoltre, il biodiesel prodotto da questa specie è conforme alla normativa ASTM 6751, lo standard statunitense per il biodiesel.

In ogni caso, uno dei principali ostacoli alla piena fruizione della produzione di lipidi da microalghe, è la capacità di estrarre l'olio dalla biomassa efficacemente e con successo. Inoltre, vi è la preoccupazione di estrarre l'olio nella maniera più sicura e ambientalmente sostenibile. In quest'ottica, un metodo di estrazione che ha guadagnato grande attenzione in questi ultimi anni è l'uso di fluidi supercritici. L'estrazione supercritica (ESC) presenta diversi vantaggi rispetto all'estrazione con solvente convenzionale, come ad esempio: le proprietà fisiche e chimiche variano molto con la temperatura e la pressione, quindi piccole variazioni possono modificare le proprietà di solvatazione, trasferimento di materia e trasmissione di calore; l'ESC produce estratti altamente puri e privi di residui di solvente potenzialmente nocivi, l'estrazione e la separazione sono veloci; il frazionamento di specifici composti è ottenibile riducendo la pressione e/o la temperatura, riducendo i costi di separazione; i composti organici sono molto solubili nei fluidi supercritici di modo che le reazioni avvengono in un'unica fase omogenea; la selettività può essere controllata variando la pressione e/o la temperatura; si può eventualmente compensare l'effetto serra utilizzando $CO_2$ di scarto dell'industria; è un'applicazione sicura per prodotti termosensibili. Tuttavia, i costi e le condizioni di esercizio gravose dei processi supercritici ne hanno limitato le applicazioni ad alcuni campi molto specialistici.

Nel presente studio, il ceppo di microalghe *Chlorella protothecoides* (UTEX 249) è stato coltivato eterotroficamente in differenti scale di laboratorio. In primo luogo, è stata studiata l'influenza di alcuni parametri, quali il mezzo di coltura, le condizioni di illuminazione, il controllo del pH e la strategia di alimentazione fed-batch; in seguito, si è operato uno *scale-up* del sistema, passando dalla coltivazione in contenitori Erlenmeyer da 250 ml a quelli da 1 litro e infine ad un comune acquario con 3,5 litri di mezzo di coltura.

La coltivazione di microalghe è stata eseguita in primo luogo in modalità batch in contenitori da 250 ml per 3 giorni a 28°C al fine di analizzare il più adatto tra due mezzi di coltura utilizzati in letteratura con risultati promettenti e di valutare l'influenza delle condizioni di illuminazione sul tasso di crescita delle microalghe. Il mezzo 1 (*medium 1*, preso da Xiong *et al.*, 2008) si è rivelato superiore rispetto al mezzo 2 (*medium 2*, preso da Chen e Walker, 2011): la differenza potrebbe essere dovuta all'effetto inibitorio della superiore concentrazione iniziale di glucosio nel mezzo 2. Per quanto riguarda l'influenza delle condizioni di illuminazione, la coltivazione al buio ha portato ad un tasso di crescita più elevato (quasi il doppio); l'assenza totale di luce potrebbe aver inibito/evitato completamente il meccanismo foto-autotrofo, assicurando soltanto la crescita eterotrofa.

Il mezzo 1 e le condizioni “al buio” sono state scelte per il primo *scale-up* a contenitori Erlenmeyer da 1 litro, con controllo del pH e strategia fed-batch. Dopo 7 giorni di coltura la concentrazione della biomassa nel mezzo era di 21,6±0,5 g/l; in confronto alla coltura in batch in contenitori da 250 ml per 5 giorni (7,9±0,5 g/l), è stata osservata una pendenza simile della curva di crescita nei primi 3 giorni e poi, a causa al consumo del contenuto iniziale di glucosio nel mezzo, la crescita in batch è terminata e si è osservata una concentrazione praticamente costante, mentre nella strategia fed-batch le microalghe hanno presentato un tasso di crescita continuo, con una pendenza praticamente costante. Inoltre, è stata osservata una produttività di biomassa di 2,87 g/l/d, molto più alta rispetto alla cultura in batch (1,58 g/l/d).

Infine, è stato eseguito un ulteriore *scale-up* a 3,5 litri di mezzo di coltura in un comune acquario fornito di una linea di alimentazione di aria compressa. La concentrazione della biomassa raggiunta è stata di 44,5 g/l in 185 ore di coltura, inferiore rispetto a quella ottenuta da Xiong *et al.* di 52,5 g/l in 168 ore di coltura, ma simile a quella ottenuta da Chen e Walker di 46 g/l. La pendenza di tutte le curve è abbastanza simile, ma nel presente studio la concentrazione iniziale della biomassa era più bassa, il che potrebbe essere stato un fattore discriminante. Inoltre, l’attrezzatura utilizzata nel presente studio è stata molto più economica, "rudimentale", anche se più difficile da controllare rispetto ad un bioreattore. In particolare, il sistema di controllo della temperatura dovrà essere più preciso in caso di ulteriori sviluppi, come per esempio l’adozione di un acquario di dimensioni inferiori e compatibili con quelle di un’incubatrice e con una termocoppia per misurare la temperatura interna costantemente. Inoltre, la portata d'aria compressa non è stata controllata con un sistema automatico, ma ciononostante il tasso di crescita è stato molto elevato. Infine, un primo *scale-up* del processo è stato raggiunto con buoni risultati. Il sistema sviluppato, sicuramente più economico rispetto ad un bioreattore automatizzato, è stato in grado di garantire una crescita su scala di laboratorio con una produttività di biomassa rapida ed elevata. Si può supporre anche un ulteriore *scale-up* su scala industriale, perché questo sistema è molto simile ad una comune vasca di ossidazione biologica per acque reflue, a condizione che sia evitata la contaminazione del mezzo di coltura, per garantire la presenza di una singola specie di microrganismo.

Per quanto riguarda il recupero dell’olio, in questo studio sono state eseguite varie estrazioni con $CO_2$ supercritica al fine di migliorare i risultati ottenuti in un precedente lavoro di Viguera *et al.* (2012). La biomassa utilizzata è stata la stessa, dato che il precedente studio è stato eseguito nello stesso laboratorio. Sono stati studiati diversi parametri, come ad esempio l'influenza della geometria del letto dell’estrattore e della portata di $CO_2$ sulla fluidodinamica e sul trasferimento esterno di materia, e l'influenza di temperatura e pressione sulla capacità di solvatazione della $CO_2$ supercritica.

Le estrazioni supercritiche sono state eseguite con un estrattore da 30 ml invece che da 100 ml, e il rapporto tra lunghezza e diametro è stato cambiato da 1:3 a 8:1. Le prove sono state eseguite a 35°C, 300 bar e 1 g $CO_2$/min. La temperatura, invece di 40°C, è stata leggermente abbassata per raggiungere una maggiore solubilità dell'olio e per ragioni di risparmio energetico.

Le estrazioni eseguite nel precedente lavoro hanno portato a solo il 6,3% in peso di olio estratto, contro il 10,9±1% in peso ottenuto in questo studio. Inoltre, il tempo di estrazione è stato drasticamente ridotto da 750 a 270 min. In più, la solubilità dell'olio nella $CO_2$ è stata drasticamente migliorata e ha raggiunto 6,8 g di olio/kg $CO_2$, confrontabile con alcuni dati di solubilità riportati in letteratura. La geometria del letto è stata identificata come il parametro discriminante, a causa di una migliore distribuzione dell'anidride carbonica supercritica nel letto fisso dell'estrattore, grazie ad un migliore rapporto tra diametro particellare e diametro dell'estrattore, che riduce la formazione di canali preferenziali e garantisce velocità lineari più elevate che hanno portato ad un maggiore coefficiente di trasferimento esterno di materia.

L'aumento della portata da 1 a 3 e 5 g/min ha condotto ad una resa di estrazione superiore e a tempi di estrazione più brevi (anche se il consumo di $CO_2$ è stato maggiore), ad un aumento della velocità lineare del processo, del numero di Reynolds e del coefficiente di trasferimento esterno di materia (questi ultimi erano in buon accordo con altri valori riportati in letteratura). La portata di 3 g/min ha portato alla resa di estrazione più elevata, ma non al tempo di estrazione più veloce, che invece è stato raggiunto con 5 g/min. In ogni caso, solo con 1 g/min la resa di estrazione è stata migliore rispetto al precedente lavoro; con le portate maggiori i risultati sono molto simili, anche se un po' più elevati nel lavoro di Viguera *et al.*. Si suppone che con la portata più bassa, la saturazione della $CO_2$ sia stata migliore che con le portate più elevate. Inoltre, è stata notata una diminuzione della solubilità dell'olio all'aumentare della portata in entrambi gli studi, e questo potrebbe essere dovuto ad un'insufficiente saturazione della $CO_2$ o alla canalizzazione nell'estrattore.

Una diminuzione della pressione ha portato alla diminuzione della solubilità dell'olio, a tempi di estrazione più lunghi e solo ad un piccolo aumento nella quantità di olio estratto. Un aumento della temperatura ha portato ad una solubilità inferiore, ma anche ad una maggiore resa di estrazione (circa il doppio), praticamente nello stesso tempo di estrazione.

Infine, un buon compromesso tra rese di estrazione elevate e tempi di estrazione brevi è stato ottenuto con una portata di 3 g/min, analogamente ai risultati ottenuti da Viguera *et al.*. Si prevede che, per migliorare il processo, si possa ottimizzare ulteriormente le condizioni di pressione e temperatura utilizzando questo valore di portata.

# Index

# Introduction

This thesis deals with a project on the extraction of oil from microalgae using supercritical $CO_2$, with the purpose of producing biodiesel from the extracted oil.

In recent years, increasing consumption and price of conventional energy, depletion of fossil fuels, global warming and greenhouse gases emissions have caused serious concerns about energy security and environmental degradation. Therefore, renewable, non-polluting biomass energy has been receiving more and more attention from both the academic community and industries. However, the use of large quantities of edible plants for the production of biofuels has raised many critics for the various issues involved (use of large areas for the intensive cultivation of cereals for human consumption, increase in their price and the consequent penalization of poor populations, low productivity of the traditional crop sources) and, for these reasons, microalgae have gained attention as a possible solution to those imminently critical issues.

Microalgae have several advantages over conventional oil crops, such as: simpler structures, but high photosynthetic efficiency and can be produced all year round; great suitability to produce biodiesel due to their high lipid content (usually in the range of 20% to 50% dry base); they may be cultivated on freshwater, saltwater lakes with eutrophication, oceans, marginal lands, deserts, etc. and there is no competition for land space that could be used for food crops; they effectively remove nutrients such as nitrogen and phosphorus, and heavy metals from wastewaters; they sequester a large amount of carbons via photosynthesis, so utilization of $CO_2$ from thermal power plants can reduce a great deal of the greenhouse gas emissions blamed for global warming.

One of the most promising microalgal species is *Chlorella protothecoides*, that was chosen for the aims of the present work. In literature, lipid contents of up to 57.8% are reported. This strain is capable to grow both autotrophically and heterotrophically; in particular, heterotrophic cultivation is regarded as the most practical and promising way to increase lipid productivity. *Chlorella* species were also reported to have high flexibility to adapt to diverse culture conditions and are likely the largest strain among microalgal species that have been tested as a biofuel feedstock. Moreover, the biodiesel produced from this species complies with ASTM 6751, the US Standard for biodiesel.

Anyway, one of the main obstacles to fully taking advantage of lipid-producing microalgae, is the ability to successfully and efficiently extract oil from the cell biomass.

Additionally, there is the concern of extracting the oil in the safest and most environmentally sustainable manner. In this view, an extraction method that has gained acceptance in recent years is the use of supercritical fluids to extract high-value products from microalgae.
Supercritical fluid extraction (SFE) presents several advantages over conventional solvent extraction, i.e. physical and chemical properties vary widely with temperature and pressure, therefore little variations can modify solvation, mass transfer and heat transmission properties; SFE produces highly purified extracts that are free of potentially harmful solvent residues (SCFs are volatile compounds at ambient conditions), extraction and separation are quick, as well as safe for thermally sensitive products (low critical temperatures); fractionation of specific compounds is feasible by lowering pressure and/or temperature, which may reduce separation costs; organic compounds are very soluble in SCFs so that the reactions take place in a single homogeneous phase; selectivity can be controlled varying pressure and/or temperature; solvent interactions can be modified by adding a modifier; possibly counteracting greenhouse gas effects by using $CO_2$ waste from industry. However, the economics and onerous operating conditions of the SFE processes has restricted the applications to some very specialized fields.

In the present study, microalgae *Chlorella protothecoides* (UTEX 249) were grown heterotrophically at different lab-scales. Firstly, the influence of some parameters was studied, such as type of growth medium, light conditions, pH control and fed-batch strategy; then, the system was scaled-up from 250-ml Erlenmeyer flasks to 1-l flask and finally to a common aquarium with 3.5 l growth medium. The aim was to optimize microalgal growth conditions, improve biomass productivity in order to reach high biomass concentrations and to develop a cheaper system, compared to an automated bioreactor. A further scale-up to an industrial scale can also be aimed, because the aquarium is very similar to a common wastewater biological oxidation tank, provided that contamination is avoided.
On the other side, various supercritical extraction assays were performed in this work in order to improve the results obtained in a previous work by Viguera *et al.* (2012). The biomass used was the same, given that the previous study was performed in the same laboratory. Different parameters were studied, such as the influence of bed geometry and $CO_2$ flow rate on fluid dynamics and external mass transfer, and the influence of temperature and pressure on the solvation capacity of supercritical $CO_2$.

The discussion has been divided into three chapters.
Chapter 1 provides a general overview on the current state of biodiesel production from microalgae, including their role in the fuel market (and not only), the various culture,

harvesting, extraction and pretreatment techniques developed in the last years, the importance of supercritical extraction as an alternative to conventional solvent extraction, the economical feasibility of a theoretical biorefinery based on microalgae and the technical limits to overcome.

Chapter 2 describes the methodology used in the laboratory experimental activity. It is divided into two sections: the first one describes the procedure followed for the heterotrophic cultivation of microalgae and the second one the procedure of supercritical extraction of oil from biomass samples.

Chapter 3 presents the results obtained from the various experiments performed both on microalgae cultivation and oil extraction with SC-$CO_2$, with a discussion over each parameter which was analyzed; furthermore, it provides a discussion and a comparison with other results from literature on the same topics.

I would like to show my thanks to the Universidad Complutense de Madrid for hosting me during my Erasmus period in Spain and my supervisor, prof. Lourdes Calvo, for following me in my internship in her laboratory. In addition, I would like to thank Javier Casas and Cristina Prieto for helping me during my work on this project.

# Chapter 1

# Biodiesel extraction from microalgae

This chapter provides a general overview on the current state of biodiesel production from microalgae, including the importance of the role of microalgae in the fuel market (and not only), the various culture, harvesting and extraction techniques developed in the last years, the importance of supercritical extraction as an alternative to conventional solvent extraction and the economical feasibility of a theoretical biorefinery based on microalgae.

## 1.1 Microalgae as a renewable feedstock

In recent years, increasing consumption and price of conventional energy, depletion of fossil fuels, global warming and greenhouse gases emissions have caused serious concerns about energy security and environmental degradation. Therefore, renewable, non-polluting biomass energy has been receiving more and more attention from both the academic community and industries. However, the use of large quantities of edible plants for the production of biofuels has raised many critics for the various issues involved, such as the use of large areas for the intensive cultivation of these plants, most of them consisting of cereals for human consumption, moral problems due to the increase in their price and the consequent penalization of poor populations, the low productivity of the traditional crop sources for the production of first and second generation biofuels.

For these reasons, microalgae have gained attention as a possible solution to those imminently critical issues. Microorganisms bear multiple advantages over traditional energy crops: they have a higher growth rate than other crops, a shorter maturity rate, a higher biomass production rate than other cash crops, as well as using far less land than conventional crops (Lee *et al.*, 2009). Furthermore, with microalgae there is no competition for land space that could be used for food crops.

Table 1.1 shows a comparison among various sources of biodiesel (Chisti, 2007); reported data were calculated to satisfy half the existing U.S. transport fuel needs by biodiesel (0.265 billion $m^3$ in 2007). As shown, this would require unsustainably large cultivation areas for major oil crops.

***Table 1.1.*** *Comparison of some sources of biodiesel* (Chisti, 2007)

| Crop | Oil yield (L/ha) | Land area needed (M ha)[a] | Percent of existing US cropping area[a] |
|---|---|---|---|
| Corn | 172 | 1540 | 846 |
| Soybean | 446 | 594 | 326 |
| Canola | 1190 | 223 | 122 |
| Jatropha | 1892 | 140 | 77 |
| Coconut | 2689 | 99 | 54 |
| Oil palm | 5950 | 45 | 24 |
| Microalgae[b] | 136900 | 2 | 1.1 |
| Microalgae[c] | 58700 | 4.5 | 2.5 |

[a] For meeting 50% of all transport fuel needs of the United States.

[b] 70% oil (by wt) in biomass.

[c] 30% oil (by wt) in biomass.

As an alternative feedstock for biodiesel production, microalgae have the following advantages over conventional oil crops such as soybeans (Wu *et al.*, 2012):

- microalgae have simple structures, but high photosynthetic efficiency with a growth doubling time as short as 24 h and can be produced all year round;
- the species abundance and biodiversity of microalgae over a broad spectrum of climates and geographic regions make seasonal and geographical restrictions much less of a concern compared with other lipid feedstocks; microalgae may be cultivated on freshwater, saltwater lakes with eutrophication, oceans, marginal lands, deserts, etc.;
- microalgae can effectively remove nutrients such as nitrogen and phosphorus, and heavy metals from wastewaters;
- microalgae sequester a large amount of carbons via photosynthesis, so utilization of $CO_2$ from thermal power plants by large-scale microalgae production facilities can reduce a great deal of the greenhouse gas emissions blamed for global warming;
- the production and use of microalgae biodiesel contribute near zero net $CO_2$ and sulfur to the atmosphere;
- microalgae can produce a number of valuable products, such as proteins, polysaccharides, pigments, animal feeds, fertilizers, and so on.

In recent years, the potential and prospect of microalgae for sustainable energy development have been extensively reviewed and microalgae are foreseen to be the fuel of the future. In fact, microalgae biofuels have been placed globally as one of the leading research fields which can bring enormous benefits to human beings and the environment (Lam and Lee, 2012).

## 1.2 Characteristics of biodiesel

Biodiesel is composed of fatty acid methyl esters (FAMEs) and is usually synthesized via transesterification of vegetable oils (triacylglycerols) with low-molecular-weight alcohols. Fatty acids are chains of carbon atoms, with or without unsaturations, which depending on their length (i.e. number of carbon atoms) and the number of unsaturations confer different properties to the various types of triglycerides.

The heating value of biodiesel produced from microalgae is reported to be 41 MJ/kg (Xu *et al.*, 2006) and complies with the US standard for biodiesel, ASTM 6571 (Li *et al.*, 2007).

In their study, Chen *et al.* (2012) explain that biodiesel generally has a higher cold filter plugging point (CFPP), density, and kinematic viscosity as well as inferior oxidation stability compared to diesel (a mixture of paraffinic, naphthenic, and aromatic hydrocarbons).

The CFPP is defined as the lowest temperature at which a given volume of biodiesel flows completely under a vacuum through a standardized filtration device within 60 s. At the CFPP, the fuel forms solids of a size that is sufficient for rendering an engine inoperable because of fuel filter plugging. Consequently, biodiesel with poor low-temperature flow properties may cause problems, such as the clogging of the fuel lines and filters in the engine fuel system, limiting the use of biodiesel in cold-weather climates.

Fuel injection systems measure fuel by volume, and thus, changes in density influence the engine output power due to the different mass of injected fuel. Thus, density is important for various aspects of diesel engine performance.

The use of fuel with a high kinematic viscosity can lead to undesired consequences, such as poor fuel atomization during spraying, engine deposits, wear on fuel pump elements and injectors, and additional energy required to pump the fuel.

Because biodiesel has an inferior oxidation stability compared to diesel because of its chemical structure, the blending of biodiesel with diesel usually causes the oxidation stability of the fuel to deteriorate. Currently, oxidation stability is one of the major technical issues in the use of biodiesel–diesel blends, especially for their long-term

storage (Chen *et al.*, 2012). The oxidation stability of biodiesels is generally related to their FAME composition and the presence of antioxidants in the feedstock. Even pure MUFA (monounsaturated fatty acid) biodiesel, although not as susceptible to oxidation as PUFA-rich fuel, will still require the addition of antioxidants to meet the limits for oxidative stability specified in the EN 14214 standard (Knothe, 2008).
Another important fuel criterium for biodiesel is bound glycerol, which functions the residual amount of triglycerides and partial glycerides in the biodiesel (Meng *et al.*, 2009).

## 1.3 Microalgae for biodiesel production

The suitability of microalgae to produce biodiesel is due to their high lipid content. In fact, the lipid content of microalgae is usually in the range of 20% to 50% (dry base), and can be as high as 80% under certain circumstances.
Selecting high lipid content and fast growing microalgae is an important step in the overall success of biodiesel production (Wu *et al.*, 2012). Table 1.2 shows the lipid content of various microalgal strains.
One of the most promising microalgal species is *Chlorella protothecoides*, that was chosen for the aims of the present work. In literature, lipid contents of up to 57.8% are reported (Table 1.2). Xiong *et al.* (2008), whose work was widely followed for the growth of microalgae in this project, also reported this result with batch culture in a 5-l bioreactor; instead, with primary and fed-batch culture they obtained lipid contents of 55.2% and 50.3%, respectively. The value of 55.2% is also reported by Xu *et al.* (2006) with *C. protothecoides*.
*Chlorella* species are robust microorganisms that can grow in many conditions around the world; they can serve as an example for heterotrophic and mixotrophic growths supplied with glucose, glycerol, acetate, or other organic compounds from waste resources with zero or negative costs as carbon source to accumulate lipids for biodiesel production (Heredia-Arroyo *et al.*, 2010).
*Chlorella* species were also reported to have high flexibility to adapt to diverse culture conditions and are likely the largest strain among microalgal species that have been tested as a biofuel feedstock that can be cultivated under phototrophic and heterotrophic conditions (Xiong *et al.*, 2008; Xu *et al.*, 2006). Chlorella is a single-celled green alga and is ellipsoidal in its shape with a diameter range from 3 to 12 μm. Heterotrophic *C. protothecoides* is composed of 40–60% lipid, 10–28% protein, 11–15% carbohydrate and 6% ash (Xu *et al.*, 2006). The fatty acid content is mainly composed of oleic, linoleic, palmitic and stearic acids (Jimenez Ruiz *et al.*, 2009).

***Table 1.2.*** *Lipid content in the dry biomass of various species of microalgae* (Wu *et al.*, 2012)

| **Species** | **Lipid content (% dry weight)** |
|---|---|
| *Anabaena cylindrica* | 4-7 |
| *Botyococcus braunii* | 25-80 |
| *Chlamydomonas reinhardtii* | 21 |
| *Chlorella emersonii* | 28-32 |
| *Chlorella protothecoides* | 57.9 |
| *Chlorella pyrenoidosa* | 2 |
| *Chlorella vulgaris* | 14-22 |
| *Crypthecodinium cohnii* | 20 |
| *Cylindrotheca sp.* | 16-37 |
| *Dunaliella bioculata* | 8 |
| *Dunaliella primolecta* | 23 |
| *Dunaliella salina* | 6 |
| *Dunaliella tertiolecta* | 35.6 |
| *Euglena gracilis* | 14-20 |
| *Hormidium sp.* | 38 |
| *Isochrysis sp.* | 25-33 |
| *Monallanthus salina* | >20 |
| *Nannochloris sp.* | 30-50 |
| *Nannochloropsis sp.* | 31-68 |
| *Neochloris oleoabundans* | 35-54 |
| *Nitzschia sp.* | 45-47 |
| *Phaeodactylum tricornutum* | 20-30 |
| *Pleurochrysis carterae* | 30-50 |
| *Porphyridium cruentum* | 9-14 |
| *Prymnesium parvum* | 22-38 |
| *Scenedesmus dimorphus* | 16-40 |
| *Scenedesmus obliquus* | 12-14 |
| *Schizochytrium sp.* | 50-77 |
| *Spirogyra sp.* | 11-21 |
| *Spirulina maxima* | 6-7 |
| *Spirulina platensis* | 4-9 |
| *Synechoccus sp.* | 11 |
| *Tetraselmis maculata* | 8 |
| *Tetraselmis sueica* | 15-23 |

The biodiesel produced from this species were acid methyl ester, linoleic acid methyl ester and oleic acid methyl ester (Gao *et al.*, 2010). Unsaturated fatty acids methyl ester comprised over 82% of the total biodiesel content (Xu *et al.*, 2006). Therefore, the properties of the biodiesel produced from *Chlorella* comply with ASTM 6751, the US Standard for biodiesel (Li *et al.*, 2007).

### 1.3.1 Metabolic pathway

Microalgae can be grown both autotrophically and heterotrophically. With the first mechanism microalgae are able to synthesize their own organic molecules from inorganic materials and using no energy deriving from the assimilated organic substances. The vast majority of autotrophic organisms are photoautotrophic because they use photosynthesis reactions, i.e. reactions that use sunlight and carbon dioxide to produce organic substances. With the heterotrophic mechanism microalgae are unable to synthesize their own food independently from inorganic substances. For survival they must therefore refer to organic compounds pre-synthesized by other organisms. On the basis of heterotrophic cultivation, researchers have carried out studies of mixotrophic cultivation which can greatly enhance the growth rate because it realizes the combined effects of photosynthesis and heterotrophy (Wu *et al.*, 2012). Mixotrophic cells harvest light and use inorganic and organic substrates as energy and carbon sources (Heredia-Arroyo *et al.*, 2010).

Heterotrophic cultivation has drawn increasing attention and it is regarded as the most practical and promising way to increase the productivity. Under autotrophic growth conditions, both biomass and lipid productivities of *Chlorella vulgaris* were low compared with those from heterotrophic growth (Liang *et al.*, 2009). *C. protothecoides* grown under autotrophic conditions had a significantly lower growth rate and lower final cell concentration than heterotrophic and mixotrophic cultures, which had the almost same specific growth rates, implying that the growth-stimulating effects of light and $CO_2$ utilization in mixotrophic cultures were not as strong as the effects of glucose (Heredia-Arroyo *et al.*, 2010).

Microalgae can adapt to different organic matters and this provides an opportunity to reduce the overall cost of microalgae biodiesel production since these organic substrates can be found in the waste streams such as animal and municipal wastewaters, effluents from anaerobic digestion, food processing wastes, etc. This aspect will be discussed below in §1.3.5.

### *1.3.2 Culture systems*

In order to achieve large-scale biodiesel production from microalgae, a cost effective cultivation system is of great significance and plays an important role to determine the successfulness of the industry (Wu *et al.*, 2012; Lam and Lee, 2012).

The cultivation systems include open and closed styles. Cultivation in an open system is usually conducted in an open pond or open tank that is widely exposed to the environment. Open ponds can be further categorized as raceway, circular, inclined and unmixed ponds. Cultivation in a closed system can be conducted in a photobioreactor, which can be further categorized into many types including tubular, vertical, flat-plate, annular, fermenter-type and internally illuminated photobioreactors. The light energy of both systems can be obtained directly from sunlight if cultivation takes place outdoors or by using an artificial light source for indoor cultivation.

An example of a raceway pond and a tubular photobioreactor are given in Figure 1.1 (Chisti, 2007) and Figure 1.2 (Suali and Sarbatly, 2012), respectively.

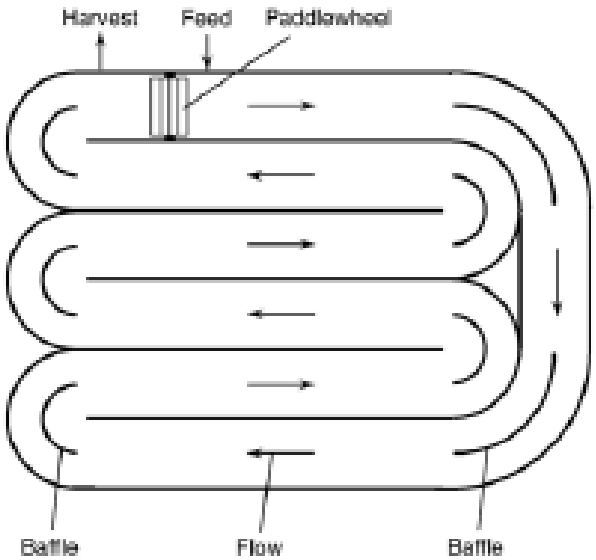


***Figure 1.1.*** *Arial view of a raceway pond* (Chisti, 2007)

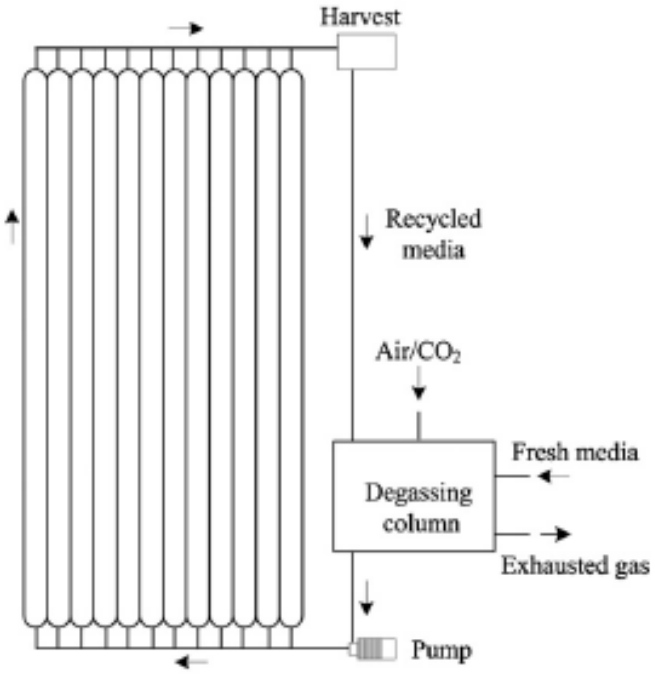


***Figure 1.2.*** *Tubular photobioreactor* (Suali and Sarbatly, 2012)

A raceway pond is made of a closed loop recirculation channel that is typically about 0.3 m deep. Mixing and circulation are produced by a paddlewheel. Flow is guided around bends by baffles placed in the flow channel. Raceway channels are built in concrete, or compacted earth, and may be lined with white plastic. During daylight, the culture is fed continuously in front of the paddlewheel where the flow begins. Broth is harvested behind the paddlewheel, on completion of the circulation loop. The paddlewheel operates all the time to prevent sedimentation. In raceways, any cooling is achieved only by evaporation. Temperature fluctuates within a diurnal cycle and seasonally. Evaporative water loss can be significant. Because of significant losses to atmosphere, raceways use carbon dioxide much less efficiently than photobioreactors. The biomass concentration remains low because raceways are poorly mixed and cannot sustain an optically dark zone (Chisti, 2007).

Although this system is relatively easy to operate and consume less energy, however high contamination level by undesired microorganisms can eventually jeopardize the survival of microalgae and affect productivity (Chisti, 2007; Lam and Lee, 2012). Cultivation in a

closed system has less contamination with the surroundings and can be easily controlled (Suali and Sarbatly, 2012).

The main advantage of growing microalgae in a closed photobioreactor is that it permits single strain culture, in which optimum growth condition is always maintained to give high consistency in biomass and lipid productivity. Thus, closed photobioreactor has always attracted great interest from researches to further improve the operating conditions for implementation in commercial scale (Lam and Lee, 2012). Anyway, compared with open pond systems, closed systems have higher capital and operational costs (Wu *et al.*, 2012).

A tubular photobioreactor consists of an array of straight transparent tubes that are usually made of plastic or glass. This tubular array, or the solar collector, is where the sunlight is captured. The solar collector tubes are generally 0.1 m or less in diameter. Tube diameter is limited because light does not penetrate too deeply in the dense culture broth that is necessary for ensuring a high biomass productivity of the photobioreactor. Microalgal broth is circulated from a reservoir (the degassing column in Figure 1.2, which is required to remove the $O_2$ that is produced during the photosynthesis process) to the solar collector and back to the reservoir (Chisti, 2007). Gas liquid mass transfer is an important feature of photobioreactors, and the biggest challenge to design the photobioreactor for high biomass productivity (Suali and Sarbatly, 2012). In fact, it is a critical factor affecting $CO_2$ utilization and hence the phototrophic growth (Wu *et al.*, 2012).

Utilizing marine microalgal strains will give benefit if large pond is used for the system. As these strains can grow in water that contains high levels of salt, they will not compete for the land already being used by other biomass-based fuel technologies. Freshwater microalgae, however, still can compete with marine microalgae if, instead of using large pond, closed photobioreactors which require less land area are used. As productivity is measured in terms of biomass produced per day per unit of available surface area, closed photobioreactors will give much higher productivities (Widjaja *et al.*, 2009).

In Table 1.3 (Chisti, 2007), a comparison between open ponds and photobioreactors is given based on an annual production level of 100 t of biomass in both cases.

In a study by Jorquera *et al.* (2010), both flat-plate photobioreactors and raceway pond showed Net Energy Ratio (NER) > 1 and are thus considered economically feasible for mass cultivation of *Nannochloropsis* for the purpose of biofuel generation. However, this study did not consider the costs required for microalgal harvesting and oil extraction, which could significantly add to the energy consumption parameter.

***Table 1.3.*** *Comparison of photobioreactor and raceway production methods* (Chisti, 2007)

| Variable | Photobioreactor facility | Raceway ponds |
|---|---|---|
| Annual biomass production (kg) | 100000 | 100000 |
| Volumetric productivity (kg $m^{-3}$ $d^{-1}$) | 1.535 | 0.117 |
| Areal productivity (kg $m^{-2}$ $d^{-1}$) | 0.048[a]<br>0.072[c] | 0.035[b] |
| Biomass concentration in broth (kg $m^{-3}$) | 4.00 | 0.14 |
| Dilution rate ($d^{-1}$) | 0.384 | 0.250 |
| Area needed ($m^2$) | 5681 | 7828 |
| Oil yield ($m^3$ $ha^{-1}$) | 136.9[d]<br>58.7[e] | 99.4[d]<br>42.6[e] |
| Annual $CO_2$ consumption (kg) | 183333 | 183333 |
| System geometry | 132 parallel tubes/units;<br>80 m long tubes;<br>0.06 m tube diameter | 978 m2/pond;<br>12 m wide, 82 m long,<br>0.030 m deep |
| Number of units | 6 | 8 |

[a] Based on facility area.

[b] Based on actual pond area.

[c] Based on projected area of photobioreactor tubes.

[d] Based on 70% by wt oil in biomass.

[e] Based on 30% by wt oil in biomass.

### *1.3.3 Influence of nutrients concentration and other parameters*

The most studied environmental variables are temperature, light and nutrients, but general trends are difficult to discern because different species respond to different variables in different ways (Stansell *et al.*, 2012).

Lipid accumulation in an oleaginous microorganism begins when it exhausts a nutrient (usually nitrogen) from the medium (Meng *et al.*, 2009). Nitrogen source and concentration in the growth media greatly influence algae lipid yield (Shen *et al.*, 2009). A high nitrogen (N) concentration is important during the first stage of the cultivation process to support the reproduction of microalgal cells, but the N concentration should be

depleted in the second stage to levels that only support the synthesis of enzymes and the critical cell formation. Thus, in the second stage, any present carbon would be converted into lipids rather than proteins, whereas protein is important for algal growth in the first stage (Suali and Sarbatly, 2012).

In nitrogen-limited situations, algae lipid content usually increases because lipid-synthesizing enzymes are less susceptible to disorganization than carbohydrate-synthesizing enzymes due to nitrogen deprivation; thus, the major proportion of carbon can be bound in lipids. However, there is usually a lipid yield peak for each algal strain at certain nitrogen concentrations (Shen *et al.*, 2009); each algal species or strain prefers different kinds of nitrogen sources (Xiong *et al.*, 2008).

In the study performed by Widjaja *et al.* (2009), higher lipid content was obtained by exposing to 7-d N starvation condition and increased further under 17-d N starvation. Interestingly, the lipid productivity was once decreased under 7-d N depletion condition and then increased back again under 17-d N starvation. Furthermore, it was found that cultivating in nitrogen depletion media will result not only in the accumulation of lipids in microalgal cells but also in gradual changing in the lipid composition from free fatty acid-rich lipids to lipids mostly containing TG (triglycerides). In the study by Shen *et al.* (2009), nitrate was the best among the three nitrogen sources for lipid production of *C. protothecoides*. Maximum lipid yield in nitrate media was at least 103% higher than that in urea media and 38% higher than that in yeast extract media. Moreover, lipid content achieved by *C. protothecoides* in low-nitrate-concentration medium was at least 23% higher than that in media with a higher nitrogen concentration, but the biomass dry weight was only 9% lower than the maximum value.

Finally, it is necessary to develop a nutrient management strategy which will first facilitate rapid biomass accumulation and then induce lipid accumulation in order to achieve maximum lipid productivity (Wu *et al.*, 2012).

The light intensity has different effects on microalgal species, as some species require more or less light energy to conduct the photosynthesis process (Suali and Sarbatly, 2012). Most experimental work on freshwater and marine microalgae was conducted under dark-light ratios of 12:12, 14:10, 10:14 and 16:8 (Jimenez Ruiz *et al.*, 2009). In the study by Heredia-Arroyo *et al.* (2010) the growth-stimulating effects of light and $CO_2$ utilization in mixotrophic cultures were not as strong as the effects of glucose and this might be an indication that *C. protothecoides 249* (the strain used in the present work) cannot utilize light in the presence of carbon sources, like an amphitrophic organism (i.e. able to live either auto- or heterotrophically).

It is well-known that dissolved oxygen (DO) is lethal to microalgal cells. However, the DO levels can be manipulated to achieve two-stage growth by controlling the concentration and dispersion rate in the culture medium. The effect of DO in the culture medium suggests that two mechanisms exist within the cell to protect the oxygen-sensitive components of nitrogenase enzyme, which is important in fixing atmospheric nitrogen to be used as a nutrient feed to microalgal growth (Suali and Sarbatly, 2012). The mechanism involves augmented respiration to scavenge the excess oxygen and a conformational state of nitrogenase that prevents damage by oxygen (Suali and Sarbatly, 2012).

Bubbling air into the *C. vulgaris* culture exerted a positive effect on cell growth (Liang *et al.*, 2009). The requirement for oxygen may be very high during the rapid growth phase of a batch culture, and oxygen limitation may result in inadequate growth and incomplete oxidation of the primary energy source. Agitation is considered one of the most important requirements in microalgae cultivation because higher biomass concentrations are produced as a result of better homogeneous distribution of nutrients and increase of air bubbles in the cultures (Heredia-Arroyo *et al.*, 2010).

The dissolved oxygen in *C. protothecoides* growth in fermentors has been generally controlled by coupling agitation speed with airflow to keep it at 20–50% air saturation (Xiong *et al.*, 2008). In flask cultures, the oxygen supply is generally provided by high agitation speeds (180 to 200 rpm) (Xu *et al.*, 2006).

### *1.3.4 Carbon sources and related issues*

To develop cost-effective algal oil production, microalgae can be cultured in heterotrophic conditions where organic carbons, such as sugars and organic acids, serve as carbon sources. This mode of culture eliminates the requirement for light and therefore, offers the possibility of greatly increased cell density and productivity (Liang *et al.*, 2009).

The most favorable glucose concentration for high lipid production is 2% (Suali and Sarbatly, 2012). Lipid contents of 57.8% were achieved when the microalgae were cultivated in a control medium with glucose not exceeding 2.4%. However, the negative influence of higher glucose concentration only appeared during the first 3 days of culture. After that time, higher glucose concentration produced higher biomass productivity in 9 days (Xiong *et al.*, 2008). In the study by Liang *et al.* (2009), under mixotrophic growth conditions, 1% and 2% glucose improved cell growth significantly compared with those at 5% and 10%. The highest glucose addition (10%) was exert inhibitory. Hence, the

substrate inhibition effect is strain-dependent and needs to be evaluated for individual study strain.

However, even though the biomass and lipid productivities are significantly higher compared with those from autotrophic growth, the cost of the organic carbon sources is high when compared against all other added nutrients. So heterotrophic microalgal cultivation may not be practical for biofuel production when the carbon source has to be purchased, as it will increase the production cost. The cost of the carbon source is one of the most discussed issues in heterotrophic growth that could hinder the success of this technique. The lipid content and biomass yield depend on the carbon type and concentration in the culture medium (Suali and Sarbatly, 2012). Glucose has been used widely as carbon source for microalgal cultivation, but the need of glucose instead of $CO_2$ for heterotrophic growth gain less interest in the view of global warming issue (Widjaja *et al.*, 2009).

Moreover, serious contamination by other microorganisms due to the presence of organic substrate can occur and the level of contamination is expected to become even more critical if wastewater is used as nutrients source. Although sterilization may provide a solution to this problem, however, high energy consumption is required and further burdens the overall process (Lam and Lee, 2012). Nevertheless, glucose which is obtained from sugar-based plant is equally important for human consumption and consequently moral issues may arise, as discussed in §1.1.

To overcome this high carbon cost, a cheap resource must be found. Crude glycerol, which is derived from biodiesel production processes as a co-product, may be capable of providing such a supply, as discussed in Liang *et al.* (2009). Using glucose derived from lignocelluloses biomass could be another alternative and low-cost carbon substrate. Also sweet sorghum is another carbon source that was reported to be suitable to culture microalgae.

### *1.3.5 Microalgae for wastewater treatment*

Due to the severe impact of chemical fertilizers towards the overall energy balance in microalgae cultivation, there is an urgent need to search for alternative and low cost nutrient sources to ensure long-term sustainability. Using wastewater to culture microalgae appears as an attractive and economical alternative. In fact, the concept of algae cultivation as engineered systems in wastewater treatment and recycling has extended the microalgae based biofuel production to its maximum potential (Li *et al.*, 2011).

Nutrients present in wastewater can be used to culture microalgae and at the same time, microalgae play an important role as reagent to purify the wastewater. In fact, algae along with many other living forms survive in the culture microenvironment developing a mutual dependence on each other and cause a reduction in the organic pollutants. The degradation also depends on the species existing (Devi *et al.*, 2012). In the last cited study, microalgae cultured in wastewater with external nutrients showed significant removal of carbon, carbohydrates and nutrients (N and P). Removal pattern of the nutrients was found to depend on the species existing and each of them was in accordance with the other.

Li *et al.* (2011) tested 14 microalgae strains in a type of wastewater called centrate, which is generated from the dewatering process of activated sludge; all 14 strains showed biomass accumulation during the 5 days of batch culture and the highest net biomass accumulation of 2.01 g/l was observed with algae strain *Chlorella kessleri* (UTEX 398), followed by *Chlorella protothecoides* (UTEX 25) with 1.31 g/l. Both strains were able to perform mixotrophic growth, in which $CO_2$ and organic carbon are simultaneously assimilated; the biomass accumulated through mixotrophic growth was higher than that accumulated through heterotrophic growth alone for both algae strains.

Finally, although there are significant advantages of using wastewater to culture microalgae, nevertheless, research in this area is still very limited. Up to now, only nearly 30% of published works on microalgae culture are using wastewater as nutrients source whereas the remaining 70% use chemical fertilizers. This is because lab-scale microalgae culture limits the use of real industrial wastewater while chemical fertilizer is easily available in the market and conveniently prepared (Lam and Lee, 2012).

### *1.3.6 Harvesting and dewatering techniques*

Microalgae cells are small (typically in the range of 2–70 μm) and the cell densities in culture broth are low (usually in the range of 0.3–5 g/l). Harvesting microalgae from the culture broth and dewatering them are energy intensive and therefore a major obstacle to commercial scale production and processing of microalgae (Wu *et al.*, 2012).

Microalgal harvesting usually involves flocculation followed by harvesting either by filtration, centrifugation, sedimentation or flotation; ultrasound techniques are still in development (Suali and Sarbatly, 2012).

Harvesting microalgae at the commercial scale usually involves a flocculant to reduce the time required to separate the medium from the algal cells. Flocculant agents are materials that have the ability to support the so-called bridging phenomena between two molecules, leading to the coagulation process. Microalgae cells always carry negative charge which

causes them to repel each other and to be suspended in liquid for a long time although mixing is not provided. By introducing coagulant that is positively charged into the culture medium, the negative charge surrounding the microalgae cells will be neutralized (Lam and Lee, 2012). Flocculants with higher molecular weights are generally more effective. High molecular weight flocculants can adsorb several particles at once, forming a three-dimensional matrix. When this occurs, the aggregated cells become easier to harvest. This is why the most effective flocculants are polymers, either natural or synthetic.

Centrifugation is the most preferred method to harvest microalgae for laboratory study. This is because this technique does not require additional chemicals; however, this method requires more electrical energy compared to flocculation. In large-scale harvesting processes, centrifugation provided good recovery and thickened the slurry, but the currently available equipment for centrifugation processes is too expensive. In contrast, the direct filtration process harvests microalgal biomass directly by using a microbial membrane which only allows algal cells to pass through (Suali and Sarbatly, 2012). This technique appears to be the cheapest technique to harvest microalgae. However, this technique requires backwashing to maintain the efficiency of the membrane filter and is time-consuming (Suali and Sarbatly, 2012).

Other new techniques under development comprise microbial flocculation (or bioflocculation), immobilization biotechnology and positively charged surface materials.

The choice of harvesting technique depends on, in part, the characteristics of microalgae (such as their size and density) and the target products (Wu *et al.*, 2012).

## 1.4 Oil extraction from microalgae

One of the main obstacles to fully taking advantage of lipid-producing microalgae, is the ability to successfully and efficiently extract oil from the cell biomass. Additionally, there is the concern of extracting the oil in the safest and most environmentally sustainable manner, therefore solvent extraction may not always be the best solution for recovering the oil from the microalgal biomass.

There are a few well-documented procedures for extracting oil from microalgae, those being mechanical pressing, homogenization, milling, solvent extraction, supercritical fluid extraction, enzymatic extractions, ultrasonic-assisted extraction and osmotic shock. All of these methods have their individual benefits and drawbacks (Mercer and Armenta, 2011).

### 1.4.1 Conventional solvent extraction

Organic solvents, such as benzene, cyclohexane, hexane, acetone and chloroform have shown to be effective when used on microalgae paste (Cravotto *et al.*, 2008; Lee *et al.*, 2009; Zheng *et al.*, 2011); they degrade microalgal cell walls and extract the oil because it has a high solubility in organic solvents. In addition, a suitable solvent should be insoluble in water, preferentially solubilize the compound of interest, have a low boiling point to facilitate its removal after extraction, and have a considerably different density than water. Also, for process cost-effectiveness, it should be easily sourced, as well as inexpensive and reusable. Due to these qualities, hexane is typically the solvent of choice for large scale extractions (Mercer and Armenta, 2011). However, n-hexane, the main component of commercial hexane, is listed as No. 1 on the list of 189 hazardous air pollutants by the US Environmental Protection Agency (Wang and Weller, 2006).

An efficient extraction requires the solvent to fully penetrate the biomass and to match the polarity of the targeted compound(s) (i.e. non-polar solvent such as hexane for extracting non-polar lipids). This, in conjunction with the ability to make physical contact with the lipid material and solvate the lipid, makes for a successful extraction solvent.

Traditionally, lipids have been extracted from biological matrices using a combination of chloroform, methanol and water (Bligh and Dyer, 1959). This procedure, known as the Bligh and Dyer method, originally designed to extract lipids from fish tissue, has been used as a benchmark for comparison of solvent extraction methods. A disadvantage of using this method is that at large scale, significant quantities of waste solvent are generated, making solvent recycling costly, as well as raising safety concerns due to handling of large amounts of organic solvents (Sahena *et al.*, 2009).

Another conventional extraction technique is called Soxhlet, which has been used for a long time. It is a standard technique and the main reference for evaluating the performance of other solid–liquid extraction methods. Soxhlet extraction is a general and well-established technique, which surpasses in performance other conventional extraction techniques except for, in limited field of applications, the extraction of thermolabile compounds. The advantages of conventional Soxhlet extraction include: (1) the displacement of transfer equilibrium by repeatedly bringing fresh solvent into contact with the solid matrix, (2) maintaining a relatively high extraction temperature with heat from the distillation flask, and (3) no filtration requirement after leaching. The main disadvantages of conventional Soxhlet extraction include (Wang and Weller, 2006):

- long extraction time;
- use of a large amount of solvent;
- agitation can not be provided in the Soxhlet device to accelerate the process;

- the large amount of solvent used requires an evaporation/concentration procedure (under vacuum);
- the possibility of thermal decomposition of the target compounds can not be ignored as the extraction usually occurs at the boiling point of the solvent for a long time.

### *1.4.2 Supercritical fluid extraction*

An extraction method that has gained acceptance in recent years is the use of supercritical fluids to extract high-value products from microalgae. This is because it produces highly purified extracts that are free of potentially harmful solvent residues, extraction and separation are quick, as well as safe for thermally sensitive products (Mercer and Armenta, 2011; Sahena *et al.*, 2009; Mendes *et al.*, 2003). Also, fractionation of specific compounds is feasible, which may reduce separation costs, as well as possibly counteracting greenhouse gas effects by using $CO_2$ waste from industry (Mercer and Armenta, 2011; Mendes *et al.*, 2003).

In a supercritical fluid extraction (SFE) system, raw plant material is loaded into an extraction vessel, which is equipped with temperature controllers and pressure valves at both inlet and outlet to keep desired extraction conditions. The extraction vessel is pressurized with the fluid by a pump. The fluid and the dissolved compounds are transported to separators, where the solvation power of the fluid is decreased by decreasing the pressure or increasing the temperature of the fluid. The product is then collected via a valve located in the lower part of the separators. The fluid is further regenerated and cycled (Wang and Weller, 2006).

Supercritical fluid extraction takes advantage of the fact that some chemicals behave as both a liquid and a gas, and have increased solvating power when they are raised above their critical temperature and pressure points. Carbon dioxide is favored because of its relatively low critical temperature (31.1°C) and pressure (72.9 atm) (Cooney *et al.*, 2009). Supercritical $CO_2$ extraction efficiency is affected by four main factors: pressure, temperature, $CO_2$ flow rate and extraction time (Sahena *et al.*, 2009).

The solubility of a target compound in a supercritical fluid is a major factor in determining its extraction efficiency. The temperature and density of the fluid control the solubility. The choice of a proper density of a supercritical fluid such as $CO_2$ is the crucial point influencing solvent power and selectivity, and the main factor determining the extract composition. It is often desirable to extract the compound right above the point where the desired compounds become soluble in the fluid so that the extraction of other compounds can be minimized. By controlling the fluid density (by increasing/decreasing

pressure) and temperature, fractionation of the extracts could also be achieved. Therefore, SFE can eliminate the concentration process, which usually is time-consuming. Furthermore, the solutes can be separated from a supercritical solvent without a loss of volatiles due to the extreme volatility of the supercritical fluid. Additionally, the diffusivity of a supercritical fluid is one to two orders of magnitude higher than that of other liquids, which permits rapid mass transfer, resulting in a larger extraction rate than that obtained by conventional solvent extractions.

The extraction time has been proven to be another parameter that determines extract composition. Lower molecular weight and less polar compounds are more readily extracted during supercritical $CO_2$ extraction since the extraction mechanism is usually controlled by internal diffusion (Wang and Weller, 2006).

These factors, along with the use of modifiers (most commonly ethanol as a co-solvent), can be altered and adjusted to optimize extractions. When ethanol is used as a co-solvent, polarity of the extracting solvent is increased and the viscosity of the fluid is subsequently altered. The resulting effect is an increase in the solvating power of the $CO_2$, and the extraction requires lower temperature and pressure, making it more efficient (Mendes *et al.*, 2003).

Since $CO_2$ is a gas at room temperature, it is easily removed when extraction is completed, thus it is safe for food applications and extraction of thermolabile compounds, and it can safely be recycled, which is an environmental benefit.

One restriction to supercritical $CO_2$ extraction is the level of moisture in the sample. High moisture content can reduce contact time between the solvent and sample. This is because microalgal samples tend to acquire a thick consistency and moisture acts as a barrier against diffusion of $CO_2$ into the sample, and diffusion of lipids out of the cells. This is why samples are dried prior to supercritical fluid extraction (Sahena *et al.*, 2009). Further downstream processing or refining is often necessary depending on what the final product is and its intended usage (Mercer and Armenta, 2011).

As SFE uses no or only minimal organic solvent (organic modifiers) in extraction, it is a more environmentally friendly extraction process than conventional solvent-solid extraction. SFE can be directly coupled with a chromatographic method for simultaneously extracting and quantifying highly volatile extracted compounds.

Finally, however, the economics and onerous operating conditions of the SFE processes has restricted the applications to some very specialized fields such as essential oil extraction, coffee decaffeination and to university research (Wang and Weller, 2006).

### *1.4.3 Pretreatment techniques*

In order to improve the extraction of oil from microalgae, namely to facilitate the penetration and diffusion of the solvent inside the matrix and/or to make the cellular lipids more accessible to it, many authors have added a further step of pretreatment of the cellular matrix; this applies to both the conventional extraction with organic solvents which, to a lesser extent, for the extraction with supercritical fluids.
Pressing and homogenization essentially involve using pressures to rupture cell walls, in order to recover the oil from within the cells. Milling on the other hand, uses grinding media (consisting of small beads) and agitation to disrupt cells. These methods are usually used in combination with some kind of solvent extraction and are based on mechanical disruption, an approach that minimizes contamination from external sources, while maintaining the chemical integrity of the substance(s) originally contained within the cells (Mercer and Armenta, 2011).
Another extraction method is the use of pulsed electric field technology (PEF), in which cells are processed by exposing them to brief pulses of a strong electric field. Electric pulses permeabilize cell walls, enhancing mass transfers across cell membranes.
An alternative, relatively safe and environmentally conscientious method involves using enzymes to breakdown cell walls of microalgal species to release cell contents (Mercer and Armenta, 2011). Enzymatic treatment of microbial biomass has the potential to partially or fully disrupt cells with minimal damage to the inside product (i.e. oil). By determining the composition of the cells being worked with, the most appropriate enzymes can be chosen to optimize extraction conditions and aim for improvements of oil yields (Mercer and Armenta, 2011).

Through cavitation, ultrasonic-assisted extractions can recover oils from microalgal cells. Cavitation occurs when vapour bubbles of a liquid form in an area where pressure of the liquid is lower than its vapour pressure. These bubbles grow when pressure is negative and compress under positive pressure, which causes a violent collapse of the bubbles. If bubbles collapse near cell walls, damage can occur and the cell contents are released (Gouveia *et al.*, 2009). Suganya and Renganathan (2012) operated, among other techniques, ultrasonication using ultrasonic probe at 24 kHz with constant temperature (50°C±1) for 5 min on macroalga *Ulva lactuca* samples obtaining the highest oil extraction yield of 8.25% (g/g), 2.25 times higher than that of direct solvent extraction. In the study of Wiyarno *et al.* (2011), ultrasonic-assisted extraction with ethanol as a solvent was performed on *Nannochloropsis* sp. and the use of ultrasonics reduced the time and temperature significantly compared to Soxhlet extraction. The GCMS test of algae oil

components indicated that there was no significant difference between both methods of extraction.

Microwave technology is based on the principle that microwave heating is very selective, and releases very little heat to the environment. Microwaves directly affect polar solvents and/or materials, so when they are used on even dried plant material, trace amounts of moisture in cells are affected. Moisture is evaporated, generating a significant amount of pressure that stresses the microorganism's cell wall, eventually rupturing it and releasing desired contents from within. Wang and Weller (2006) explain that solvent choice for microwave-assisted extraction (MAE) is dictated by the solubility of the extracts of interest, by the interaction between solvent and plant matrix, and finally by the microwave absorbing properties of the solvent determined by its dielectric constant, which should be high. The extracting selectivity and the ability of the solvent to interact with microwaves can be modulated by using mixtures of solvents. One obvious drawback of using microwaves is the potential for oxidative damage to valuable lipid products (Sahena *et al.*, 2009). In addition, microwave extraction of microbial oils could still prove difficult to scale up.

Many applications of this novel technique can be found in literature. Lee *et al.* (2009) extracted oil from various microalgal species using a slightly modified version of Bligh and Dyer's method with various pretreatment techniques; MAE resulted the best extraction technique. Dejoye *et al.* (2011) compared extraction yields and fatty acid profiles from freeze-dried *Chlorella vulgaris* by microwave pretreatment followed by supercritical carbon dioxide extraction (MW-$SCCO_2$) with those obtained by supercritical carbon dioxide extraction alone ($SCCO_2$). Experiments performed at 28 MPa and 40°C using MW-$SCCO_2$ gave the highest extraction yield and the highest concentrations of fatty acids compared to $SCCO_2$ extraction without pretreatment. A recent study by Balasubramanian *et al.* (2011) was successful in demonstrating that MAE of *Scenedesmus obliquus* can give oil recoveries of 77% compared to 47% for the control Soxhlet extraction. The major advantage of this type of extraction is the 20-fold reduction in processing time; additionally, the quality of the resulting oil was better than that of the control or the Soxhlet extraction, containing more unsaturated fats, as well as more omega-3 and omega-6 fatty acids.

Both ultrasound and microwave-assisted methods improve extractions of microalgae significantly, with higher efficiency, reduced extraction times and increased yields, as well as low to moderate costs and negligible added toxicity (Mercer and Armenta, 2011).

### *1.4.4 Biochemical and thermochemical conversion*

The conversion of microalgae to biofuel can be classified as either a biochemical and thermochemical conversion process (Suali and Sarbatly, 2012). The biochemical conversion processes of biofuel are transesterification and fermentation, which produce biodiesel and ethanol as the main products, respectively. The thermochemical processes can be categorized as pyrolysis, liquefaction, gasification and hydrogenation. The pyrolysis and liquefaction processes produces bio oil fuel as the main product, whereas gasification produces syngas and hydrogenation is a process to improve the biofuel or feedstock properties.

Transesterification requires 3 mol of alcohol for each mole of triglyceride to produce 1 mol of glycerol and 3 mol of methyl esters (Figure 1.4; Chisti, 2007). The reaction is an equilibrium. Industrial processes use 6 mol of methanol for each mole of triglyceride (Fukuda *et al.*, 2001). This large excess of methanol ensures that the reaction is driven in the direction of methyl esters, i.e. towards biodiesel. Yield of methyl esters exceeds 98% on a weight basis. Transesterification is catalyzed by acids, alkalis and lipase enzymes. Alkali-catalyzed transesterification is about 4000 times faster than the acid catalyzed reaction. Consequently, alkalis such as sodium and potassium hydroxide are commonly used as commercial catalysts at a concentration of about 1% by weight of oil. Alkoxides such as sodium methoxide are even better catalysts than sodium hydroxide and are being increasingly used (Chisti, 2007). Use of lipases offers important advantages, but is not currently feasible because of the relatively high cost of the catalyst (Fukuda *et al.*, 2001; Chisti, 2007).

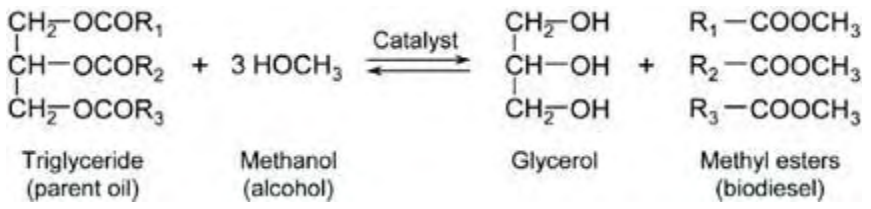


***Figure 1.4.*** *Transesterification of oil to biodiesel* (Chisti, 2007)

Alkali-catalyzed transesterification is carried out at approximately 60°C at atmospheric pressure. Under these conditions, reaction takes about 90 min to complete. Methanol and oil do not mix, hence the reaction mixture contains two liquid phases. Other alcohols can be used, but methanol is the least expensive. To prevent yield loss due to saponification

reactions (i.e. soap formation), the oil and alcohol must be dry and the oil should have a minimum of free fatty acids. Biodiesel is recovered by repeated washing with water to remove glycerol and methanol (Chisti, 2007).

***Table 1.4.*** *Main characteristics of pyrolysis, thermochemical liquefaction and gasification of microalgae* (Suali and Sarbatly, 2012)

| **Parameter** | **Pyrolysis** | **Liquefaction** | **Gasification** |
|---|---|---|---|
| Feedstock conditions | Biomass should be free from water content | Tolerable moisture content of biomass up to 65% | Moisture less than 40% |
| Pretreatment of feedstock | Drying microalgae biomass | Pretreatment with catalyst in surge bin to make microalgae slurry | - |
| Preferable catalyst | - | Ni based catalyst | Dolomite, nickel, potassium carbonate |
| Operation temperature | 300-750°C | 200-500°C | 0-700°C |
| Operation pressure | Atmospheric | 2-25 MPa | Atmospheric |
| Main product yield | Bio oil: 28.6-57.9% (fast pyrolisis)<br>Pyrolysis gas: 13-25% (slow pyrolysis) | Bio oil | $H_2$: 5-56%<br>CO: 9-52% |
| Energy content of main product | Bio oil: 30.7-41 MJ/kg<br>Pyrolyisis gas: 1.2-4.8 MJ/kg | 30-39 MJ/kg | - |
| Co-products | Bio char: 10-25% (slow pyrolysis) | Methane (6-20%) with 21 MJ/kg heating value | $CH_4$ and other HC products, $CO_2$, tar: up to 20%, ash |
| Recyclable waste | Fluidizing gas can be recycled to pyrolysis reactor) | Aqueous co-product can be used as feed to microalgae culture | - |

The pyrolysis process of microalgal biomass is an anaerobic heating process that does not involve oxidation and occurs at high temperatures between 200 and 750°C. Pyrolysis can be classified into two main categories: fast or slow pyrolysis. The thermochemical liquefaction of biomass is a process that requires heating the biomass at high temperatures ranging from 200 to 500°C with pressures greater than 20 bar in the presence of a catalyst. Gasification of microalgae is a process in which the carbonaceous compounds of the biomass react with air, steam or oxygen at high temperatures ranging from 200 to 700°C in a gasifier and involves other thermochemical processes such as pyrolysis and combustion. This results in production of clean $H_2$; methane can be considered to be a co-product and is only produced in small amounts (Suali and Sarbatly, 2012). Hydrogenation of a carboneous feedstock is the process of adding or reacting $H_2$ into the double bonds of hydrocarbons. This reaction results in the production of better feedstocks for other process or products that have lower molecular weights.
The main characteristics of pyrolysis, thermochemical liquefaction and gasification of microalgae is reported in Table 1.4 (Suali and Sarbatly, 2012).

## 1.5 Economical feasibility

Biodiesel production from microalgae is an emerging technology considered by many as a very promising source of energy, mainly because of its reduced competition for land. However the impact assessment and the energy balance performed by Lardon *et al.* (2009) show that algal biodiesel suffers from several drawbacks at the current level of maturity of the technology. In comparison to conventional energetic crops, high photosynthetic yields of microalgae significantly reduce land and pesticide use but not fertilizer needs. Moreover, production, harvesting, and oil extraction induce high energy consumption, which can jeopardize the overall energetic balance. The authors conclude that even if the algal biodiesel is not really environmentally competitive under current feasibility assumptions, there are several improvement tracks which could contribute to reduce most of its impacts.
In the study by Amer *et al.* (2011), the scale up and commercialization of 5 algae-to-biofuels processes from a quantitative perspective was considered. The production costs calculated were several orders of magnitude greater than the selling price of oil from seed crops. It was shown that each process had multiple hurdles to overcome and price reductions to achieve before algae-based biofuels were economically competitive with traditional fossil oil or other oil crops. The open pond scenarios were closest to the $1/kg price point, and at this moment appear to be closest to commercialization. Bioreactor-

based growth methods were shown to currently be prohibitively expensive, but future technological advances may drastically improve the economic outlook for these scenarios. Brentner *et al.* (2011) identified as the best scenario the following sequence: flat panel photobioreactor, flocculation with Chitosan, direct transesterification by supercritical methanol, anaerobic digestion, which had an 85% savings relative to the base case, i.e. open raceway pond cultivation, centrifugation, drying and hexane extraction of the oil, standard transesterification with methanol, and landfilling of residual biomass. But even the best scenario had a slightly negative net energy balance.

Tabernero *et al.* (2012) described a promising plant of biodiesel covering all production system. The biomass came from a heterotrophic culture of microalgae, extracting the oil with supercritical fluids. With a surface of only 7500 $m^2$ it would be possible to manufacture 10000 ton of biodiesel per year. The economic evaluation was calculated with several assumptions, obtaining a manufacture costs bigger than the soy-based biodiesel and with a high investment, but without economical and social problems in connection with the raw material. This evaluation also revealed that to make this type of plant viable, residues must be commercialized, with the subsequent develop of a microalgae business market. However, with conservative assumptions, the plant would never be viable due to bioreactors expenses.

In Figure 1.5 a summary of microalgal transformations into biofuel is shown.

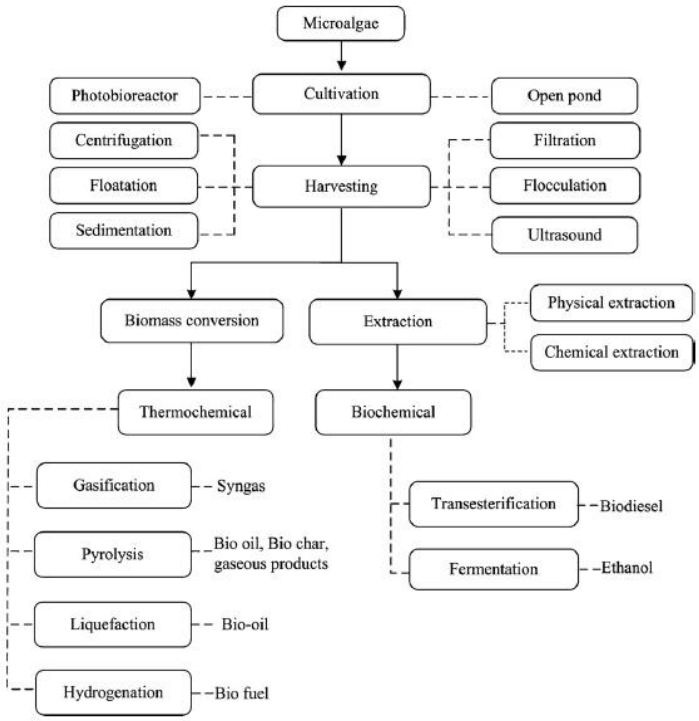


***Figure 1.5.*** *Summary of microalgal transformations into biofuel. The main product of each process is shown at the end of the transformation route* (Suali and Sarbatly, 2012)

In both conventional and supercritical extractions, one of the most energy and cost consuming steps is the dewatering of microalgal paste by centrifugation and specially thermal drying, strictly necessary in SFE as described above in §1.4.2. In SFE also the system costs are not negligible (due to high pressures), while in conventional extraction the great amounts of solvent used and its separation costs are also high.

Xu *et al.* (2011) reported that the drying step consumed about 25% of total energy input. They also compared a "dry" with a "wet route" showing that the total energy input was approximately the same for both routes (even if they had to make some assumptions as regards the wet route because it can not be stated to what extent dewatering was necessary and/or economical) and concluding that improving mechanical drying as far as possible and use the best available technologies for thermal drying was very important to reduce energy consumptions and, as a consequence, oil extraction costs.

To overcome the dewatering problem, some authors operated extractions on wet microalgae; for example, Yoo *et al.* (2012) started from a wet algal biomass with more than 99% water content and performed oil extraction with osmotic shock (using NaCl or sorbitol) and a mixture of methanol-hexane as solvent. Furthermore, Balasubramanian *et al.* (2011) started from a wet algae to water ratio of 1:1 g/g (84.8% moisture content) and extracted more lipids than control with a continuous microwave processing system, using hexane as solvent.

As regards supercritical extraction, it appears clear that it is necessary to start from dried microalgae, because water is an obstacle for supercritical $CO_2$, as described in §1.4.2. Even if Halim *et al.* (2012) reported a more successful extraction with wet algae paste (30% wt. solid concentration) than with oven-dried and milled algae biomass.

In an innovative process by OriginOil (Eckelberry *et al.* 2009), on which a patent is still pending, the algae solution is channeled through a pipe to which an electromagnetic field and ultrasound are applied, rupturing the cell walls and releasing the oil. Carbon dioxide is bubbled through, which lowers the pH. The resulting solution is then piped into another container. The lowered pH separates the biomass from the oil, and the oil floats to the top, while the biomass sinks to the bottom. The oil can be skimmed off, the biomass can be further processed, and the water is recycled.

Finally, a large-scale plant for production of biodiesel from microalgae could be possible in the future, more research should be done in different alternative fields to improve the process until higher benefits and a complete viability is obtained.

## 1.6 Aim of the thesis

The objectives of this project were to firstly cultivate heterotrophically a promising lipid-producing microalgae strain, *Chlorella protothecoides* (UTEX 249), with the aim to develop a cheap culture system if compared to an automated lab-scale bioreactor. Various parameters, such as type of medium, influence of light/dark conditions and different scales were studied, as described later in Chapter 2. Secondly, various supercritical $CO_2$ extractions were performed in this work in order to improve the results obtained in the previous work by Viguera *et al.* (2012), using the same biomass but varying type of extractor (volume), its geometry (length to diameter ratio) and operative conditions (temperature, pressure, flow rate).

# Chapter 2

# Materials and methods

This chapter describes the methodology used in the laboratory experimental activity. It is divided into two sections: the first describes the procedure followed for the heterotrophic cultivation of microalgae and the second the procedure of supercritical extraction of oil from biomass samples.

## 2.1 Culture of microalgae

The strain of microalgae was *Chlorella protothecoides* (UTEX 249), due to its high lipid productivity and its capability to grow both in autotrophic and heterotrophic conditions. It was purchased from UTEX center The Culture Collection Of Algae - University Of Texas at Austin.
As discussed in Chapter 1, heterotrophic growth is considered the most promising way to grow microalgae for the production of biodiesel; so this metabolic pathway was chosen in the present work.

### *2.1.1 Culture media*

For the heterotrophic culture of microalgae, two different types of culture medium were tested. The first one was taken from the work of Xiong *et al.* (2008) (medium 1), the second one from the work of Chen and Walker (2011) (medium 2).
Medium 1 was composed of the following compounds described in Table 2.1. Medium 2 was composed of the following compounds described in Table 2.2.

***Table 2.1.*** *Composition of culture medium 1*

| Name | Formula | Quantity | Unit |
|---|---|---|---|
| Potassium di-Hydrogen Phosphate | $KH_2PO_4$ | 0.70 | g/l |
| Di-Potassium Hydrogen Phosphate | $K_2HPO_4$ | 0.30 | g/l |
| Magnesium Sulphate Heptahydrate | $MgSO_4 \cdot 7H_2O$ | 0.30 | g/l |
| Ferric Sulfate Heptahydrate | $FeSO_4 \cdot 7H_2O$ | 3.00 (1.00) | mg/l (ml/l) |
| Vitamin B1 – Thiamine Hydrochloride | | 0.01 (1.00) | mg/l (ml/l) |
| Glucose | $C_6H_{12}O_6$ | 15.00 | g/l |
| Glycine | $H_2NCH_2COOH$ | 0.10 | g/l |
| A5 Trace Mineral Solution | | 1.00 | ml/l |
| Yeast Extract | | 4.00 | g/l |

***Table 2.2.*** *Composition of culture medium 2*

| Name | Formula | Quantity | Unit |
|---|---|---|---|
| Potassium di-Hydrogen Phosphate | $KH_2PO_4$ | 0.70 | g/l |
| Di-Potassium Hydrogen Phosphate | $K_2HPO_4$ | 0.30 | g/l |
| Magnesium Sulphate Heptahydrate | $MgSO_4 \cdot 7H_2O$ | 0.30 | g/l |
| Ferric Sulfate Heptahydrate | $FeSO_4 \cdot 7H_2O$ | 3.00 (1.00) | mg/l (ml/l) |
| Vitamin B1 – Thiamine Hydrochloride | | 0.01 (1.00) | mg/l (ml/l) |
| Glucose | $C_6H_{12}O_6$ | 30.00 | g/l |
| Calcium Chloride | $CaCl_2 \cdot H_2O$ | 0.025 (1.00) | g/l (ml/l) |
| Sodium Chloride | NaCl | 0.025 (1.00) | g/l (ml/l) |
| A5 Trace Mineral Solution | | 1.00 | ml/l |
| Yeast Extract | | 4.00 | g/l |

Potassium di-Hydrogen Phosphate, Di-Potassium Hydrogen Phosphate, Magnesium Sulphate Heptahydrate, Glycine, D(+)-Glucose and Yeast Extract (Cultimed) were provided by Panreac (Spain). Sodium Chloride, extra pure, was provided by Scharlau Chemie s.a. (Spain).

The A5 trace mineral solution had the following composition (Table 2.3):

***Table 2.3.*** *Composition of A5 trace mineral solution*

| Name | Formula | Quantity | Unit |
|---|---|---|---|
| Boric Acid | $H_3BO_3$ | 2.9 | g/l |
| Manganese Chloride | $MnCl_2 \cdot 2H_2O$ | 1.8 | g/l |
| Zinc Sulphate Heptahydrate | $ZnSO_4 \cdot 7H_2O$ | 0.22 | g/l |
| Copper Sulfate Pentahydrate | $CuSO_4 \cdot 5H_2O$ | 0.08 | g/l |
| Molybdenum Trioxide | $MoO_3$ | 0.018 | mg/l |

The initial pH of medium 2 was adjusted to 6.8 as described in the relative work; medium 1 was kept with its initial pH of about 6.3.

The first step was to pour approximately 100 ml of Milli-Q water in a one-liter glass container and to calibrate the balance. Subsequently, the compounds listed in Table 1 or 2 had to be added. $FeSO_4 \cdot 7H_2O$, $CaCl_2 \cdot H_2O$, NaCl, Vitamin B1 and A5 solution were poured through a 1 ml syringe, the other compounds with a spoon. Finally, the container was filled with Milli-Q water up to 1 l, shaken to homogenize the culture medium and a piece of autoclave tape was applied on the cap.

The two most important components of these media proposed by both Xiong *et al*. (2008) and Chen and Walker (2011) were the quantities of glucose and yeast extract in the medium, which are sources of carbon and nitrogen respectively and promote cell growth.

### *2.1.2 Preparation and sterilization of equipment*

All the equipment used for microalgae culture and handling had to be previously sterilized in an autoclave Med 12 (J.P. Selecta, Spain) at 121°C for 20 minutes. The equipment involved included culture medium, glucose solution, KOH solution (Technical, ≥85%, powder, Sigma-Aldrich, India), syringe tips, compressed air line and Erlenmeyer flasks.

While performing sterilization, the cap of culture medium, glucose solution and KOH solutions was not completely closed to avoid formation of vacuum. Once sterilized, they were closed in order to prevent their contamination. Syringe tips were put in the appropriate box sealed with tape and aluminum sheet to prevent water infiltration before sterilization. The compressed air line for aeration contained a filter (< 0.20 μm, Sartorius 17805 E, Spain) and ended in a glass pipette that bubbled the air into the cultures. For sterilization glass pipettes were wrapped in an aluminum sheet held by a piece of tape to prevent water infiltration and the plastic tubes of the air line put into a glass container (the

cap was not completely closed). Erlenmeyer flasks were sealed with cotton wrapped in gauze and with aluminum foil on the top to prevent water infiltration.
For the culture of a greater volume of biomass (about 4-5 l), an aquarium was used. It could not be sterilized in the autoclave because of its size, so it was firstly cleaned with isopropanol and then sterilized by 10 minutes of UV irradiation in a vertical laminar flow cabinet Telstar Mini-V/PCR.
The vertical laminar flow cabinet was sterilized by means of isopropanol and subsequent 10 minutes of UV irradiation; firstly, the cabinet had to be started turning on the fan. This operation must be done every time before using the cabinet to prevent contamination of the working environment and of the equipment. It is important to note that, in order to avoid contamination of the working environment, only the equipment that was strictly necessary was placed under the cabinet, especially if it was not sterile.
Before starting any operation it was necessary to wear sterile gloves and wash the hands with isopropanol. At the end of any operation, all the contaminated equipment used had to be washed with bleach or autoclaved before binning.

### 2.1.3 Culture of microalgae in Petri dishes

In this culture technique, a solid culture medium was used instead of the liquid media described above. Thus it was necessary to add 15 g/l of bacteriological agar (Agar, Bacteriological European Type, Panreac, Spain) to fresh liquid medium and then to sterilize it in the autoclave.
About 15-20 ml of agar medium was added to each Petri plate in the vertical laminar flow cabinet. Once the medium had solidified, fresh microalgae inocula were added. Two different techniques were utilized:

- Plating by streaking: an inoculum was taken with a sterile inoculation loop from a tube containing fresh microalgae and transferred into the Petri dish. The lid of the Petri dish to inoculate was lifted to about a 45 degree angle and the inoculating loop containing the microalgae sample was streaked across the surface of about one quarter of the solidified agar. This operation was repeated four times until the whole agar surface was covered; the first two streaks were thicker than the last two, to observe colonies of different sizes.
- Plating by grid: the procedure was the same as described above, with the difference that the microalgae sample was streaked across the surface as to from a grid.

Inoculated Petri dishes were then sealed and put in an oven at 28°C for about two weeks. Once the cultures were grown, they were sealed with laboratory film and stored in a refrigerator to interrupt their growth (Figure 2.1).


***Figure 2.1.*** *Chlorella protothecoides grown in Petri dishes*

### *2.1.4 Batch culture of fresh microalgae in 250-ml Erlenmeyer flasks*

First, 100 ml of medium were poured into each 250-ml Erlenmeyer flask; then the fresh microalgae inoculum was taken with a sterile inoculation loop by scraping it gently over the surface of a microalgae colony to obtain some of the material and transferred into the Erlenmeyer flask. An important shrewdness was to always keep the cap in the hand and close the container (Erlenmeyer flask and microalgae tube) every time an operation was performed, to avoid contamination. This procedure was valid for all types of microalgae growth techniques described below.

Subsequently, Erlenmeyer flasks were put in an orbital incubator (Gallenkamp, UK) that worked at 28°C and 200 rpm for 4 days. Every day the growth of microalgae was monitored by means of the optical density at 540 nm using the Shimadzu UV-1800 spectrophotometer, as described later.

### 2.1.5 Batch culture of Petri dish microalgae in 250-ml Erlenmeyer flasks

The procedure of culture of Petri dish microalgae was almost the same as that described in the previous paragraph, with the difference that inocula came from microalgae grown for two weeks in Petri plate and stored in a refrigerator, as described above.
Two different conditions were tested to evaluate the influence of light: in one case microalgae were cultured in the presence of light (no direct solar irradiation), in another case the incubator was sealed by means of aluminum sheets and microalgae were cultured in dark conditions. In batch culture of fresh microalgae only the first condition was tested.

### 2.1.6 Batch culture of microalgae in 1-l Erlenmeyer flasks

Working in the sterilized vertical laminar flow cabinet, to each 1l-flask about 400 ml of medium were added and 100 ml inoculum from the 250-ml flasks. In this case, the orbital incubator was too small and a static incubator Memmert 2010 (Spain) was used and oxygenation was provided by means of compressed air.
Culture conditions were the same as for smaller Erlenmeyer flasks: 28°C for 4 days. Every day the growth of microalgae was monitored by means of the optical density at 540 nm.

### 2.1.7 Fed-batch culture of microalgae in 1-l Erlenmeyer flasks with pH-control

In order to scale up the process another culture step in 1-l Erlenmeyer flasks was performed, in order to reach a greater biomass concentration and production.
The procedure adopted was almost the same as that described in the previous paragraph, but to improve the process it was necessary to feed microalgae and to regulate the acidic environment, by means of daily glucose addition and control of pH of the culture. The culture period was longer as in batch culture and reached 7 days.
A 300 g/l glucose solution was used, in order to reduce the large quantities that had to be fed and avoid excessive dilution. Glucose solution quantity was calculated taking into account that 20 g/l had to be fed to each culture every day.
To control pH and keep its value above 6.0, a solution of KOH (10 g/l) was used and the quantity to add every day was established according to the acidity of the culture medium.
The feeding of both solutions was performed by means of a syringe working in the flow cabinet.

### 2.1.8 Fed-batch culture of microalgae in aquarium with pH-control

The culture of a large volume of biomass was performed in a common aquarium provided of compressed air line (with a filter) and a thermostat to control the temperature of the culture medium (Figure 2.2). Working in the cabinet, 3 l of fresh medium was inoculated with 600 ml Erlenmeyer culture. Temperature was adjusted to 28ºC with an error of ±1.5ºC. Culture was performed in dark conditions.

***Figure 2.2.*** *Chlorella protothecoides grown in aquarium with fed-batch strategy and pH control*

Glucose feeding and pH-control were performed every day for the whole culture period. The quantity of glucose added was 20 g/l/d. pH was kept above 6.0 by feeding of a 50 g/l KOH solution.
Silicone antifoaming liquid (Panreac, Spain) was added to prevent excessive foaming.

### 2.1.9 Biomass recollection and drying

At the end of incubation, microalgae were harvested in centrifuge containers and centrifuged at 9000 rpm for 10 min at ambient temperature (Centrifuge Sigma 4-16K, Germany).
The centrifuged microalgae paste was separated by the liquid phase by means of a syringe. The wet biomass was dried in an oven at 50°C to constant weight.

### 2.1.10 Spectrophotometry

The growth of microalgae was monitored by increase of the optical density at 540 nm using the Shimadzu UV-1800 spectrophotometer. The correlation between optical density and biomass concentration was obtained from the weight of dried microalgae. The following correlation was obtained (Figure 2.3):

$$y = 0.384x \ (R^2 = 0.998) \qquad (2.1)$$

where:

- y = biomass concentration (g/l)
- x = absorbance at 540 nm

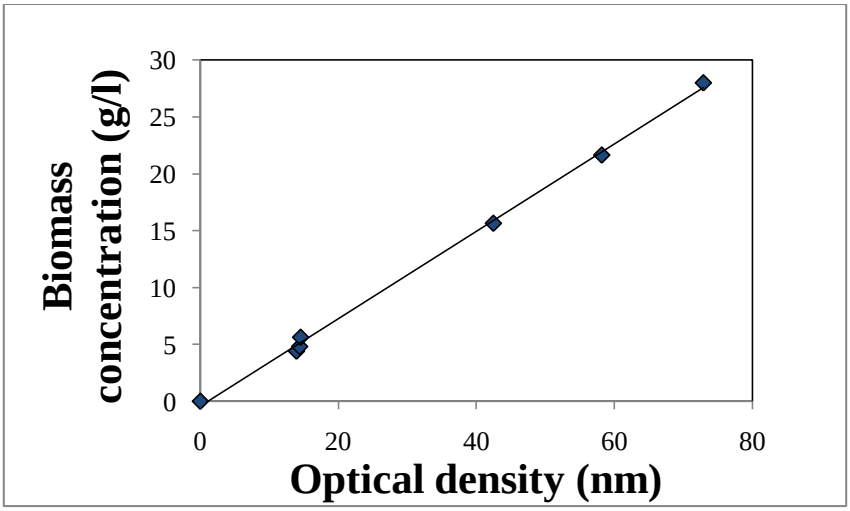


***Figure 2.3.*** *Correlation between optical density and biomass concentration*

For optimal measurements, samples were diluted to obtain spectrophotometric readings in the range of 0.2 to 0.8. To the diluted samples a correction factor was applied, as illustrated in the following example: to a sample of culture medium of **a** ml which is diluted by adding **b** ml of purified water, the following correction must be applied:

$$D_{540nm-T} = D_{540nm-0} \cdot x - D_{540nm-B} \quad (2.2)$$

$$x = 1 + \frac{b}{a} \quad (2.3)$$

where:

- x = correction factor
- $D_{540nm-T}$ = corrected optical density value
- $D_{540nm-0}$ = optical density value of the sample (0.2-0.8)
- $D_{540nm-B}$ = optical density value of culture medium before inoculation

## 2.2 Supercritical extraction

Supercritical extraction equipment and operating procedure are described below. SFE was chosen in the present work because of its advantages over conventional extraction described in Chapter 1.

### *2.2.1 Supercritical extraction equipment*

In Figure 2.4 the extraction equipment is shown. The high purity $CO_2$ (99.9% by Carburos Metalicos) was fed from the pressured container (60 bar) in the liquid phase, then cooled in a temperature-controlled bath (Selecta, Frigiterm-30) to -15°C and impelled by a cooled-head membrane pump (Jasco-PU 2080). This refrigeration prevented pump cavitation in the pressurisation process.

The pump had a pressure control system within that allowed to stop its activity to detect that the limit was exceeded.

Carbon dioxide at high pressure coming out from the pump was heated to the process conditions. This was performed by locating around the extractor a heating jacket with a temperature control of ±1°C and recorded by a type K thermocouple placed inside the reactor in direct contact to the fluid and the solid. The heating jacket was also used to pre-heat part of the solvent feeding line. Pressure was read by a Bourbon manometer with an accuracy of ± 5 bar.

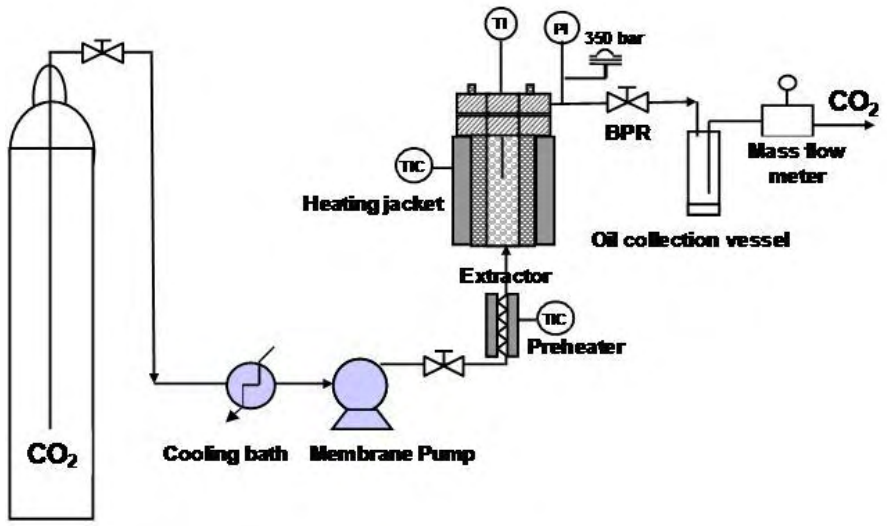


***Figure 2.4.*** *Supercritical fluid extraction equipment*

The extractor consisted of a SS316 vessel of approximately 30 ml total capacity, in which the biomass to treat was placed as to form a fixed bed. The control of pressure and $CO_2$ flow rate was achieved through the combined action of the back pressure regulator (BPR) valve (Tescom, Series 26-1700) and the flow regulator of the pump. This regulation valve underwent a drastic change of pressure within it, thus had to be kept at high temperature to avoid the formation of carbon dioxide plugs, therefore heating jacket was placed around it which avoided these drawbacks.

As a protective measure, the equipment was provided with a relief valve calibrated to 350 bar which avoided overpressure problems.

At the exit from the BPR valve, $CO_2$ was a gas at ambient conditions and all the soluble compounds were recollected in a container that displayed the progress of the extraction. Finally, the amount of $CO_2$ per unit of time (or the total amount) was recorded by means of a mass flow meter (Alicat Scientific, M-10SLPM-D) connected to the end of the line with an accuracy of ± 0.5 g/min.

### 2.2.2 Operating procedure

Dry biomass, previously weighed and adequately pre-treated (grinded in Moulinex A505 during three cycles of 15 s), was loaded into the extractor, forming a fixed bed. The quantity of biomass loaded was about 3 g. Subsequently, the extractor was closed and pre-heated. After that, the $CO_2$ was pumped in, and, once the desired pressure was reached, the back pressure regulator (BPR) valve was opened, providing a continuous

flow through the bed. After the valve, the $CO_2$ was depressurised, the solvent power of the supercritical fluid dropped and the extract precipitated in a previously weighed glass flask. When the established treatment time was over, the apparatus was depressurised, the extract weighed and the biomass unloaded.

At each established time interval (5-10-15 min depending on the duration of the experiment), the container was weighed in order to obtain the extraction curve. The extraction curves were constructed by plotting the extracted oil to biomass ratio (wt%) versus the amount of $CO_2$ which passed through the bed to obtain that oil amount at constant intervals of time.

The operative conditions tested were 150 and 300 bar, 35 and 70°C, 1, 3 and 5 g $CO_2$/min in various combinations, in order to study their influence on the extraction yield and time.

# Chapter 3

# Results and discussion

This chapter presents the results obtained from the various experiments performed both on microalgae cultivation and oil extraction with SC-$CO_2$, with the procedures described in the previous chapter.

## 3.1 Culture of microalgae

The various microalgae culture techniques described in the previous Chapter 2 were performed in order to obtain an improvement in biomass production compared with other results that can be found in previous works on the same topic. In particular, one of the aims was to reproduce and further improve the good results obtained by Xiong *et al.* (2008) and Chen and Walker (2011) in their studies on the culture of *Chlorella protothecoides*, one of the most promising microalgae strains because of its high lipid content if cultivated under heterotrophic conditions.

### *3.1.1 Medium selection*

In the primary investigation, two different growth media were compared, as described in §2.1.1. The differences were that the initial pH of medium 2 was adjusted to 6.8, as described in the study by Chen and Walker (2011), and both media had similar but slightly different compositions (Tables 2.1 and 2.2, §2.1.1). Medium 2 has a higher initial glucose content (double) with respect to medium 1.

As shown in Figure 3.1, microalgae grown in medium 1 had a higher biomass concentration during the whole culture period, till reaching the concentration of 7.0±0.5 g/l after 70 hours. Microalgae grown in medium 2 only reached 5.4±0.5 g/l after 72 hours. No substrate feeding and no pH control during the cultivation period were operated. The difference could be due to the inhibitory effect of the higher initial glucose concentration in medium 2; in fact, Xiong *et al.* (2008) investigated different initial glucose concentrations (15, 30, 45, 60 g/l) and noticed that higher glucose concentrations negatively influenced cell growth, and this was reflected by a lower specific growth rate within the first 72 h of culture. According to the above observation, continuous feeding of

glucose at lower concentration, which was proved helpful to cell growth and lipid accumulation, was adopted as a basic strategy for their subsequent fed-batch assays.

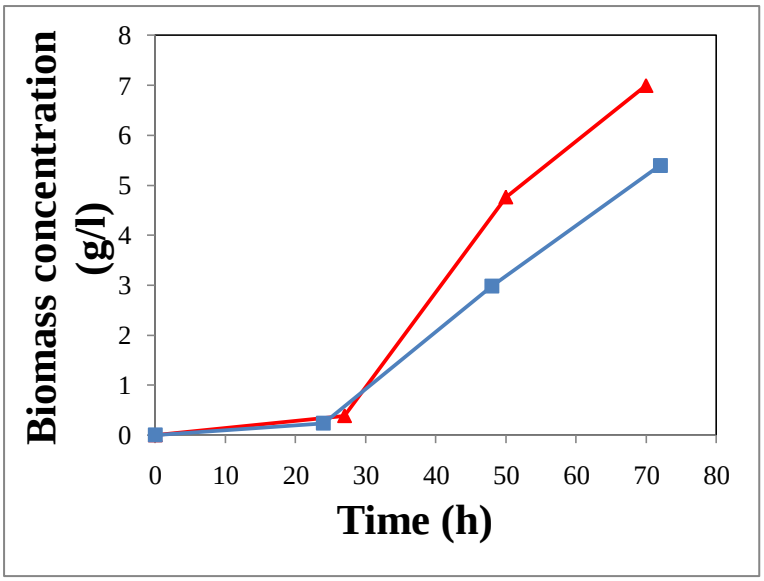


***Figure 3.1.*** *Batch culture in 250-ml Erlenmeyer flasks: comparison of culture media. Red: medium 1, Blue: medium 2*

From these first experiments medium 1 seemed to be superior in comparison to medium 2, and was consequently chosen for the scale up of the process to 1l-Erlenmeyer with fed-batch and pH-control strategy, as described below in §3.1.3.

### *3.1.2 Influence of light*

The influence of light was tested in 250-ml Erlenmeyer cultures. As shown in Figure 3.2, dark conditions gave a better result than light conditions in medium 1, because the concentration reached was about twice that of the latter; the growth rate was also faster, as can be seen from the slopes of the two growth curves. Microalgae cultivated in medium 1 in light conditions and in dark conditions reached 4.0±0.5 g/l and 7.7±0.5 g/l, respectively, in 4 days of incubation at 28°C and 200 rpm.

In the study by Heredia-Arroyo *et al.* (2010) the growth-stimulating effects of light and $CO_2$ utilization in mixotrophic cultures were not as strong as the effects of glucose and this might be an indication that *C. protothecoides 249* cannot utilize light in the presence of carbon sources, like an amphitrophic organism (i.e. able to live either auto- or

heterotrophically). Therefore, dark conditions might be the only way to avoid photoautotrophy and ensure heterotrophic growth for this strain of microalgae. Chen and Walker used only dark conditions, whereas Xiong *et al.* used weak light conditions (5 μmol $m^{-2}$ $s^{-1}$).

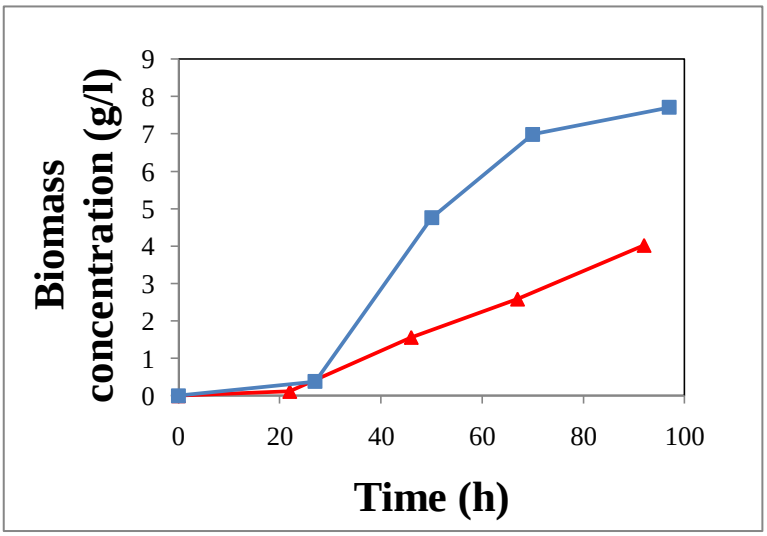


***Figure 3.2.*** *Batch culture in 250-ml Erlenmeyer flasks: influence of light. (Diamonds) medium 1 in light conditions, (squares) medium 1 in dark conditions, (triangles) medium 2 in dark conditions.*

In both the conditions tested, in the first 24 hours of culture growth curves had almost the same behavior and the biomass concentrations reached were practically similar; subsequently, the influence of light condition could be appreciated and strongly influenced microalgae growth.
Dark conditions were chosen for further scale-up of the process and fed-batch strategy.

### *3.1.3 Fed-batch and pH-control*

In order to scale-up the process a culture step in 1-l Erlenmeyer flasks with glucose feeding and pH-control was performed, in order to reach a greater biomass concentration and production.
The culture period was longer than in batch culture, about 7 days, because of substrate feeding which allowed to keep microalgae in the exponential growth phase and reach a higher biomass concentration.

After 7 days of culture in dark conditions biomass concentration was 21.6±0.5 g/l, as displayed in Figure 3.3. Comparing to batch culture in 250-ml flasks for 5 days (7.9±0.5 g/l) in dark conditions, a similar slope can be observed in the first 3 days; then, due to the consumption of the initial glucose content in the medium, growth in batch culture ended and quite constant biomass concentration can be observed, whereas in fed-batch strategy microalgae presented a continuous growth rate. A similar trend can be observed in Chen and Walker (2011) and Xiong *et al.* (2008): substrate feeding led to continuous cell growth with practically a constant slope, whereas batch culture led to substrate depletion and constant biomass concentration.

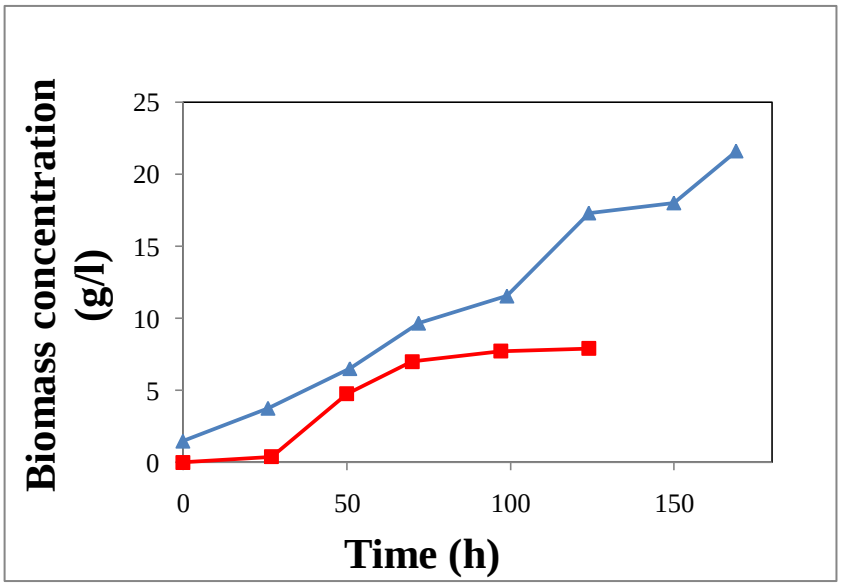


***Figure 3.3.*** *Scale-up of the process and fed-batch strategy. Blue: fed-batch culture in 1-l Erlenmeyer flasks with pH control; Red: batch culture in 250-ml Erlenmeyer flasks without pH control*

In fed-batch strategy, a 300 g/l glucose solution was used and a quantity of 20 g/l/d was added. The pH was regulated with a 10 g/l KOH solution to keep its value above 6.0. In fact, every day pH lowered to values between 5 and 6, with minimum values in the order of 4.5, and was raised to 6.5-6.8 adding the required amount of KOH solution. In the study by Xiong *et al.*, a value of 6.5 was considered as optimal for *Chlorella protothecoides* growth.

In this first scale-up of the process, a biomass productivity of 2.87 g/l/d was observed, much higher than in batch culture (1.58 g/l/d).

### *3.1.4 Scale-up of the process*

The culture of a large volume of biomass was performed in a common aquarium provided of compressed air line. Three liters of fresh medium were inoculated with 600 ml batch culture. Results are shown in Figure 3.4, where the growth curves obtained by Xiong *et al.* (2008) and Chen and Walker (2011) in fed-batch culture in a 5-l working bioreactor were reported as a comparison. The biomass concentration reached was of 44.5 g/l in 185 h of culture, lower than that obtained by Xiong *et al.* of 52.5 g/l in 168 h of culture, but similar to that obtained by Chen and Walker of 46 g/l. The slope of all the growth curves is quite similar; however, in this study the culture started from a lower initial biomass concentration (1.23 g/l instead of 5 g/l in Xiong *et al.*) and that could have been a discriminating factor.

Moreover, the equipment used in the present study was much more cheaper, "rudimental", though more difficult to control than a bioreactor. In particular, temperature control was performed with a thermostat which was not calibrated for the volumes of medium, but temperature values were fairly constant at 28±1.5°C; it was reported that *C. protothecoides* grows better in temperatures between 25 and 28°C (Xu *et al.*, 2006; Xiong *et al.*, 2008; Heredia-Arroyo *et al.*, 2010). For further developments, anyway, a more controlled and precise temperature control system will definitely be necessary, as for example a smaller size aquarium compatible with the size of an incubator and with an internal thermocouple to measure temperature constantly.

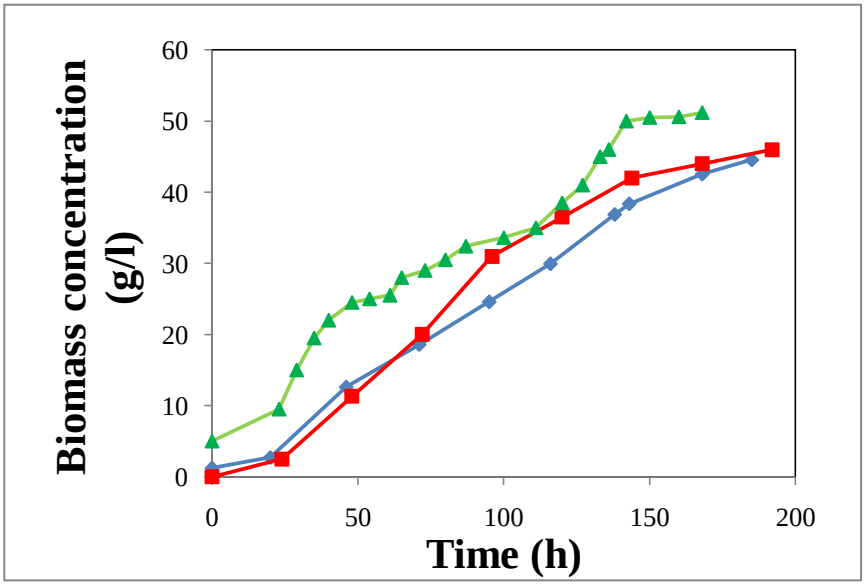


***Figure 3.4.*** *Fed-batch culture in aquarium in dark conditions and with pH control. Blue: aquarium; Green:* Xiong *et al.* (2008)*, improved fed-batch strategy; Red:* Chen and Walker (2011)*, fed-batch fermentation.*

In both studies by Chen and Walker and Xiong *et al.*, dissolved oxygen in the medium was kept between 20-50% and over 40%, respectively, by automatically controlling agitation speed and air flow rate in the bioreactor. In the present study, compressed air flow rate was not controlled with any automatic system, but nevertheless growth rate was very good. In previous experiments, with 5 to 8 l of medium instead of 3.5, this parameter affected microalgae growth. In fact, using a rather low air flow rate, the biomass concentrations reached were very low and growth curves presented an "oscillating" behavior, meaning that part of the biomass died and glucose feeding and pH control were insufficient to microalgae growth. On the other side, an increased air flow rate caused the formation of a high amount of foam; but it was well controlled by adding a larger quantity of silicone antifoaming. Another problem that was solved by using an increased air flow rate was excessive dilution of the culture; in fact, daily evaporation was insufficient and large volumes (till 8 liters) were early reached, leading to a very difficult control of microalgae growth.

Finally, a first scale-up of the process was achieved with good results. The aim was to develop a cheaper system, compared to an automated bioreactor, to grow microalgae on a lab-scale with high and fast biomass productivity. A further scale-up to an industrial scale can also be aimed, because this system is very similar to a common wastewater biological oxidation tank, provided that contamination is avoided.

## 3.2 Supercritical extraction

Various supercritical extraction assays were performed in this work in order to improve the results obtained in the previous work by Viguera *et al.* (2012). The biomass used was the same, given that the previous study was performed in the same laboratory.

### *3.2.1 Factors affecting fluid dynamics and external mass transfer: effect of bed geometry and $CO_2$ flow rate*

Recasens and Velo (2001) explain that the interest in mass transfer in high-pressure systems is related to the extraction of a valuable solute with a compressed gas, and that the solute is either a volatile liquid or solid deposited within a porous matrix. The compressed fluid density, especially in supercritical state, approaches a liquid-like value, so the solubility of the solute in the fluid can be substantially enhanced over its value at low pressure. Moreover, the retention mechanism of the solute in the solid matrix is only

physical (that is, unbound, as with the free moisture), or strongly bound to the solid by some kind of link (as with the so-called bound moisture). Crushed vegetable seeds are an example of both retention mechanisms; in fact, they have a fraction of free, unbound oil that is readily extracted by the gas, while the rest of the oil is strongly bound to cell walls and structures and this bound solute requires a larger effort to be transferred to the solvent phase.
The extraction of soluble compounds from solid plant material proceeds in several steps (Brunner, 1999):

1. The plant matrix absorbs the supercritical solvent and other fluids that are deliberately added to influence the extraction process.
2. In parallel to step 1, the extracted compounds are dissolved by the solvent. A chemical reaction may occur prior to solvation.
3. The dissolved compounds are transported to the outer surface of the solid. Diffusion is the most important transport mechanism.
4. The dissolved compounds pass through the outer surface.
5. The compounds are transported from the surface layer into the bulk of the supercritical solvent and are subsequently removed with the solvent from the bulk of the solid material. Similar sequences apply to the SFE of other solid materials.

Recasens and Velo (2001) explain that beside the solubility behaviour, mass-transfer plays an essential role for extraction processes. At the beginning, the extraction efficiency is limited by the solubility in the available amount of fluid. Higher solubilities, and therefore shorter extraction times, can be achieved by increasing the extraction pressure, which increases the fluid density and consequently the solvent power. The same is normally found also by increasing the extraction temperature, because at higher pressure levels the increase in vapour pressure of the substances to be dissolved is more effective than the reduction in the fluid density at higher temperatures. A reduced density of 2.22 is required for maximizing the solubility of triglycerides, the major constituents of seed oils, in a critical fluid (King, 2002).
In Figure 3.5 a typical trend of extraction-yield over extraction-time is shown.
The second phase of extraction is controlled by diffusion, and this, especially, leads to long extraction times. Therefore, the aim is to reach the proposed extraction yield within the solubility phase, because otherwise the process will not be economical (Recasens and Velo, 2001).

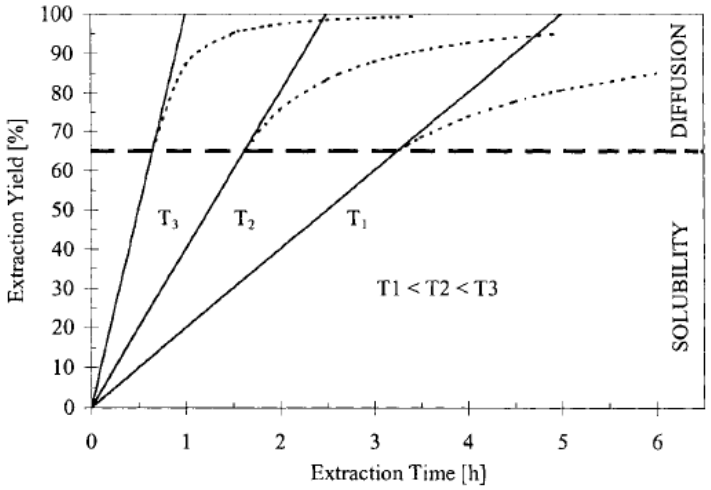


***Figure 3.5.*** *Typical trend of extraction curves* (Recasens and Velo, 2001)

Supercritical $CO_2$ extractions were performed at various pressures, temperatures and $CO_2$ flow rates over dried and grinded microalgal biomass samples obtained from a previous work by Viguera *et al.* (2012), that contained an oil percentage of 35% dry weight.

In contrast with Viguera *et al.*, extractions were performed in this work with a different extractor having a smaller volume and a different bed geometry. In fact, instead of using an extractor of 100 ml of volume, a 30 ml extractor was used; moreover, the bed geometry was radically changed from L/D = 0.33 to L/D = 8, in order to greatly increase the linear velocity of the process, maintaining about the same time of contact.

In comparison with the study by Viguera *et al.*, assays at 35°C, 300 bar and 1 g $CO_2$/min were performed. Temperature, instead of 40°C, was slightly lowered in order to reach a greater solubility of microalgae oil and for energy saving reasons, when thinking on an industrial scale up of the process. Anyway, operative conditions were practically the same.

As shown in Figure 3.6, extractions performed in the previous work led to only 6.3%wt. oil extracted in contrast with 10.9±1%wt. in the extractions performed with the smaller extractor. Moreover, the extraction time was drastically reduced from 750 min to 270 min. Also, oil solubility in carbon dioxide was drastically improved as can be seen by the slope of the extraction curves.

Extractions were performed at different temperatures, but the difference in oil solubility was too great to be only explained by this parameter. Bed geometry was identified as the discriminating parameter, because of a better distribution of supercritical carbon dioxide in the fixed bed of the extractor due to a better particle diameter to extractor diameter

ratio ($d_p/d$) that reduced the formation of preferential channels and higher linear velocities (u, m $s^{-1}$) that led to higher external mass transfer coefficients ($k_g$, m $s^{-1}$). This will be discussed below.

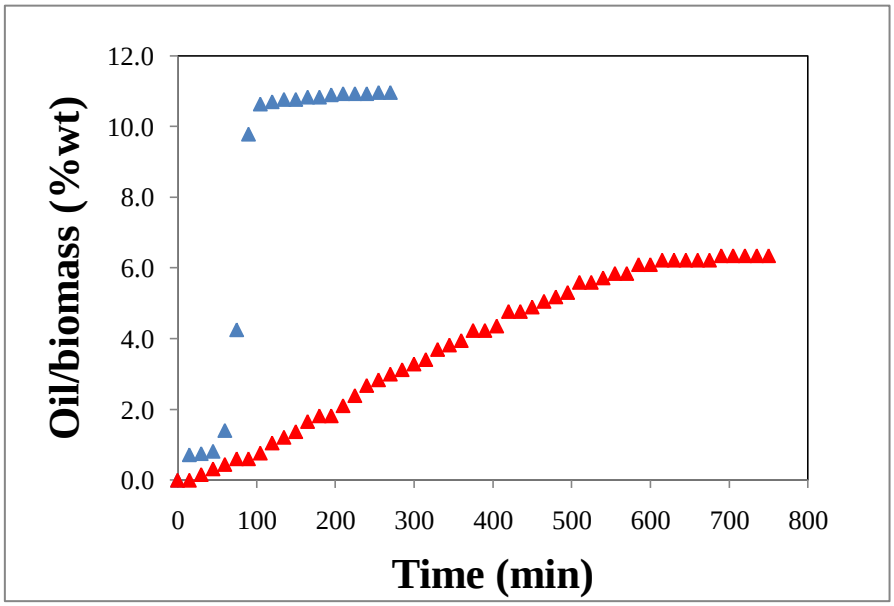


***Figure 3.6.*** *Extractions performed at 1 gCO2/min. Blue: present work, 35°C and 300 bar; Red:* Viguera *et al., 40°C and 300 bar.*

The effect of the increase of $CO_2$ flow rate was studied, in comparison with the study by Viguera *et al.*. The same values were used, i.e. 3 and 5 g/min. Results were shown in Figures 3.7 and 3.8. An increase in flow rate led to a higher extraction yield and to shorter extraction times, even if the consume of $CO_2$ was larger. The flow rate of 3 g/min had the highest extraction yield but not the fastest extraction time, which was achieved with 5 g/min. The same behavior can be observed in the assays by Viguera *et al.* (Figure 3.8).
Results are summarized in Table 3.1. As can be seen, only with 1 g/min $CO_2$ flow rate the extraction yield was better than in the previous work; with higher flow rates results are very similar, although a little bit higher in the work by Viguera *et al.*. It is suggested that with the lowest flow rate used, $CO_2$ saturation with oil was better than with the higher flow rates. Bed geometry also was suggested to have a great influence, as discussed previously.

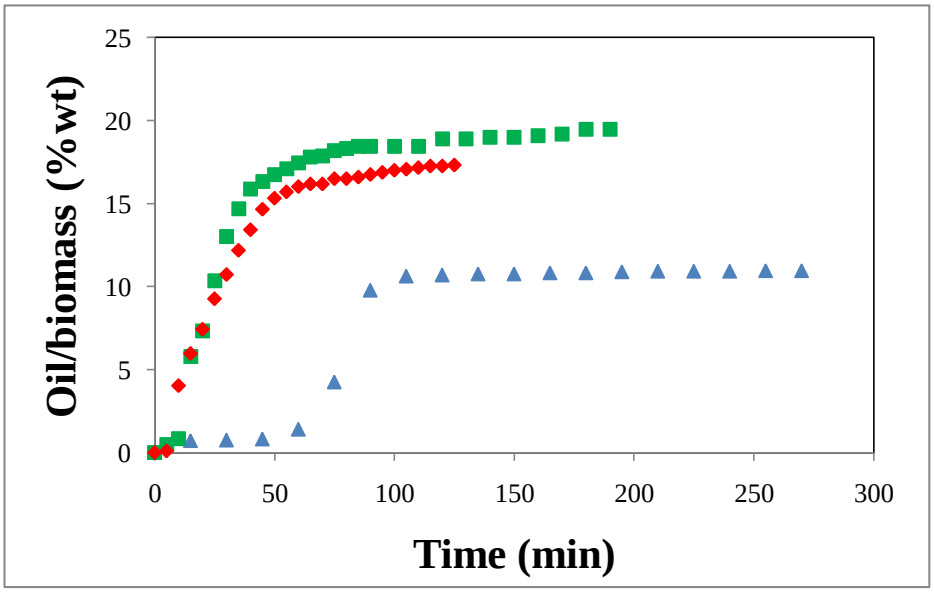


***Figure 3.7.*** *Effect of $CO_2$ flow rate at 35°C and 300 bar. Blue: 1 g/min; Red: 3 g/min; Green: 5 g/min*

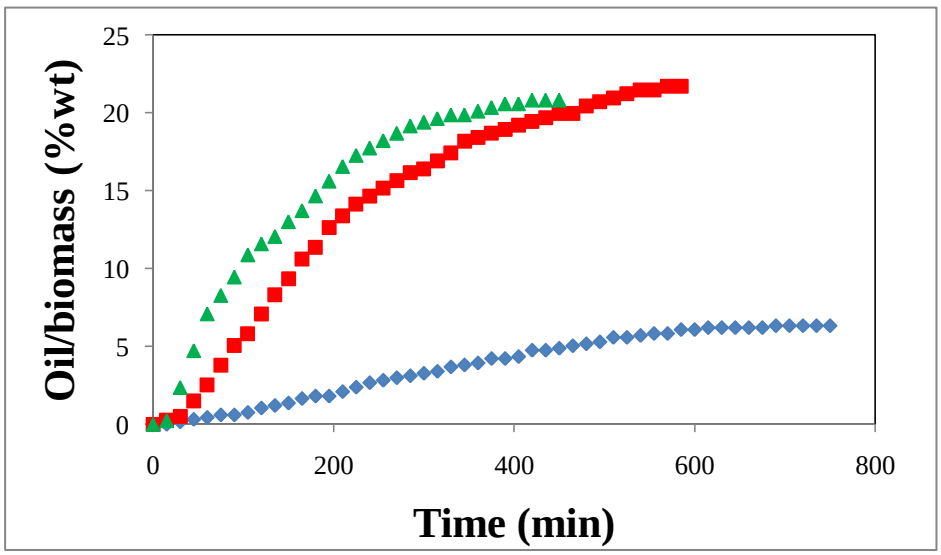


***Figure 3.8.*** *Effect of $CO_2$ flow rate at 40°C and 300 bar. Blue: 1 g/min; Red: 3 g/min; Green: 5 g/min* (Viguera *et al.*, 2012)

Anyway, in the present work, solubilities and in particular extraction times were noticeably improved (Table 3.1). Solubility should be constant because the same T and P

conditions are maintained, but a decrease is noticed as the flow rate increases. In the study by Mateus *et al.* (2011), it was proved that the fatty acid profile of oil extracted from *C. protothecoides* is very similar to that of olive oil (variety Arbequina). Anyway, it is difficult to find solubility data of microalgae oil in literature; assuming that the oil extracted in this study should have solubility values similar to those of olive oil, only 6.8 g/kg (Table 3.1) is in accordance with some reported values; in fact, in Blasco *et al.* (2004) solubility of olive oil at 280 bar and 35°C was 6.23 g/kg. At 300 bar and 35°C solubility should be higher. The values obtained at higher flow rates are inferior, and this might be due to an insufficient saturation of $CO_2$ with oil or to channeling in the extractor.

***Table 3.1.*** *Influence of CO2 flow rate on extraction parameters*

| **Study** | **Flow rate (g/min)** | **Extraction yield (%wt)** | **Extraction time (min)** | **Oil solubility (g oil/kg $CO_2$)** |
|---|---|---|---|---|
| Present study | | | | |
| | 1 | 10.9±1 | 270 | 6.8 |
| | 3 | 19.5±1 | 190 | 4.6 |
| | 5 | 17.3±1 | 125 | 1.9 |
| Viguera *et al.* (2012) | | | | |
| | 1 | 6.3 | 750 | 0.5 |
| | 3 | 21.7 | 585 | 1.1 |
| | 5 | 20.8 | 450 | 1.0 |

The increase in oil yield, on the other side, is a consequence of the increase in flow rate. In fact, both the Re number and the mean concentration gradient ($\Delta c_m$) are increased by higher flow rates; in the first case a higher rate implies a higher velocity and therefore a higher Re number (with constant extractor diameter). The mean concentration gradient increases because the equilibrium concentration is constant at a given T and P, and the loading of the fluid is lower (Lack *et al.*, 2001).

As shown in Table 3.2, the increase in flow rate had the following consequences:

- an increase in the linear velocity of the process (u), Re number and external mass transfer coefficient ($k_g$);

- a decrease in contact time.

The increase of length to diameter ratio (L/D) in the extractor, also had important consequences (Table 3.2):

- an increase in linear velocities, Re numbers and external mass transfer coefficients in all the flow rate conditions tested;
- contact times were more or less of the same order of magnitude.

***Table 3.2.*** *Influence of bed geometry and $CO_2$ flow rate on external mass transfer and contact time*

| L/D | Mass rate (g $min^{-1}$) | u ($x10^3$ m $s^{-1}$) | Re | Contact time (s) | $k_g$ ($x10^5$ m $s^{-1}$) | $k_g \cdot a \cdot \rho$ (kg $s^{-1}$ $m^{-3}$) |
|---|---|---|---|---|---|---|
| 1/3 | 1 | 0.026 | 8 | 385.9 | 0.6 | 72.9 |
| 1/3 | 3 | 0.078 | 23 | 128.6 | 1.14 | 138.4 |
| 1/3 | 5 | 0.130 | 38 | 77.2 | 1.54 | 187.1 |
| 8/1 | 1 | 0.228 | 21 | 350.2 | 1.12 | 138.4 |
| 8/1 | 3 | 0.685 | 64 | 116.7 | 2.14 | 265.1 |
| 8/1 | 5 | 1.142 | 107 | 70.0 | 2.9 | 359.2 |

To calculate the mass transfer coefficient the following correlation by Wakao and Kaguei (1982) was used:

$$\frac{Sh}{\sqrt[3]{Sc}} = \frac{2}{\sqrt[3]{Sc}} + 1.1Re^{0.6}$$

where Sh is the Sherwood number; Sc is the Schmidt number and Re is the Reynolds number. The external mass transfer coefficient, $k_g$ in m $s^{-1}$, was deduced from the Sh number:

$$Sh = \frac{k_g d_p}{D_e}$$

where $d_p$ is the particle diameter and $D_e$ is the effective diffusivity of supercritical carbon dioxide. As shown in Figure 3.9, the calculated values are in good agreement with other reported values. The external mass transfer coefficient can be influenced by the diffusion coefficient, which is also included in the Sh number, as in the Sc number. Diffusion can be increased by shortening the diffusion length. For solid materials this is achieved by smaller particle sizes, which further leads to a higher specific interfacial area. However, there is a limit for reducing the particle size because if the particles are too fine, the problem of channeling arises, so an optimum has to be found (Lack *et al.*, 2001).

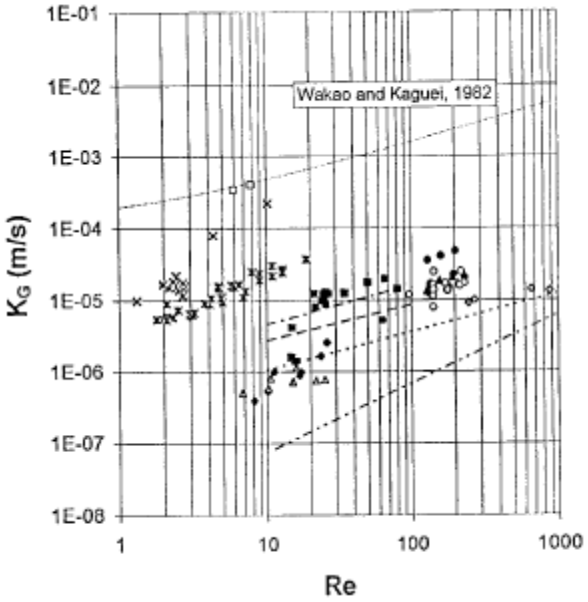


***Figure 3.9.*** *Comparison of the overall, gas-based, mass-transfer coefficient for several liquid-to-gas and solid-to-gas packed beds and column systems* (Puiggené *et al.*, 1997)

*- Fixed Beds: (□) (♦) (*) (X) (._.) (---)*
*- SFE Columns: (●) (σ) (μ) (ν) (Γ)*
*- Low pressure columns: (...) (_.._..)*

Density and viscosity of SC-$CO_2$ at various conditions of temperature and pressure were calculated using the NIST Chemistry WebBook. The particle diameter had a distribution in the range between 0.1 and 0.5 mm; an average value of 0.3 mm was taken for calculations.

The effective diffusivity value was taken from Fiori (2009); in the cited study, oil was extracted from sunflower seeds with SC-$CO_2$ and a value of $3.3x10^{-11}$ $m^2$ $s^{-1}$ was reported for effective diffusivity. The seeds were milled to obtain different particle sizes with a mean diameter between 0.19 and 1.2 mm, thus, similar to the mean value used in the present work. Moreover, the value used for $D_e$ is in accordance with Del Valle *et al.* (2006).

Finally, the influence of solvent ratio on oil extraction rate is shown in Figure 3.10. With increasing solvent ratios, the extraction rate increased, but loading of the supercritical solvent decreased at high solvent ratio (corresponding to 5 g/min), due to the short residence time of the solvent. At the solvent ratio corresponding to 3 g $CO_2$/min, the extraction rate was maximal and was achieved after 30 min of extraction.

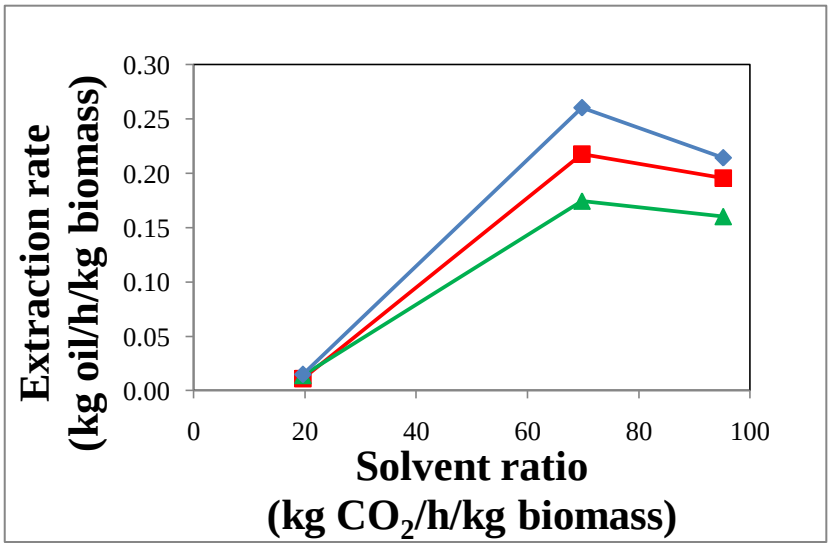


***Figure 3.10.*** *Dependence of extraction rate on solvent ratio at 300 bar and 35°C. Blue: 30 min, Red: 45 min,Green: 60 min*

## *3.2.2 Operative conditions affecting solvation capacity of $CO_2$: effect of pressure and temperature*

Thermodynamic and transport properties of Supercritical Fluids (SCFs) are very peculiar, because they are intermediate between those of a gas and a liquid. In the case of carbon dioxide ($CO_2$), as illustrated in Figure 3.11, at temperatures below $T_c$ (304.1K) a liquid-vapor equilibrium and a discontinuity in the density of both phases exists. Increasing the

temperature, the difference between the density of both phases decreases and vanishes upon reaching the critical temperature. Near the critical point, the density rapidly increases with pressure. At constant pressure, the density decreases with increasing temperature. In the blue area the density oscillates between 100 and 800 kg/m$^3$, therefore the density of supercritical $CO_2$ (SC-$CO_2$) is closer to that of a liquid than that of a gas. The solvent power of a SCF depends on its physical state (defined by pressure, temperature and density) and on its chemical nature (polarity, acid-base properties, etc.). Since normally SC-$CO_2$ density is between 600-900 kg/m$^3$, its solubility parameter (Hildebrand parameter) is similar to that of conventional organic solvents like acetone or n-hexane.

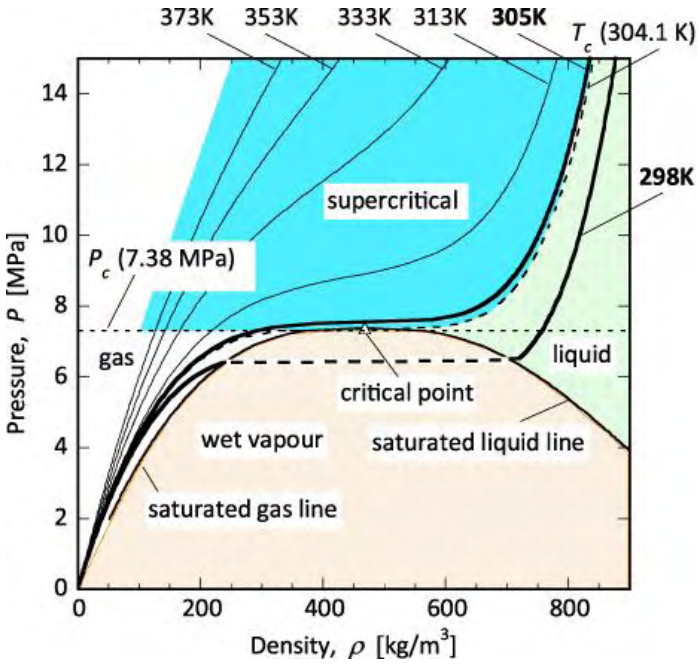


***Figure 3.11.*** *Phase diagram (P,ρ) of $CO_2$*

An important parameter in operations where a SCF acts as solvent is the solubility of a compound in the SCF. Low volatility compounds dissolved in SCFs present a higher concentration than that determined by Raoult's law, of several orders of magnitude. The increase of vapor pressure of a condensed phase by hydrostatic pressure is known as *Poynting effect*; it becomes significant only from 100 MPa and therefore has relevance in operations with SCFs. On the other side, low volatility compounds dissolved in SCFs present a behaviour known as *inversion of the variation of solubility*. At high pressures, solubility increases with temperature and vice versa. This phenomenon is due to two

opposite effects: solvent power increases with density and vapor pressure increases exponentially with temperature. Chemical nature and structure of both the gas and the compound to be dissolved influence the solubility of a compound in a SCF; since SC-$CO_2$ behaves like an apolar solvent it has greater affinity for apolar solutes. Moreover, the solubility of a compound depends on the chain length, ramification, number of rings and substituents, interactions among the solutes (i.e. mixing of reactants) (Brunner, 1994).

In Figure 3.12 results of the variation of operative conditions are shown. Taking the extraction at 300 bar, 35°C and 1 g/min as a reference, a decrease in pressure led to lower oil solubility (as expected, Figure 3.13), longer extraction times and only a small increase in the quantity of extracted oil. In particular, the extraction time was about 2.5 times longer and the increase in extraction yield only of 2% (Table 3.3).
An increase in temperature led to a lower solubility but also to a higher extraction yield (about two times) in practically the same time of extraction. The decrease in oil solubility was not so marked as in the assay at lower pressure (Figure 3.13), but was unexpected because this would mean that the crossover pressure is at pressures higher than 300 bar. In fact, at high pressures, solubility should increase with increasing temperature, as described before.

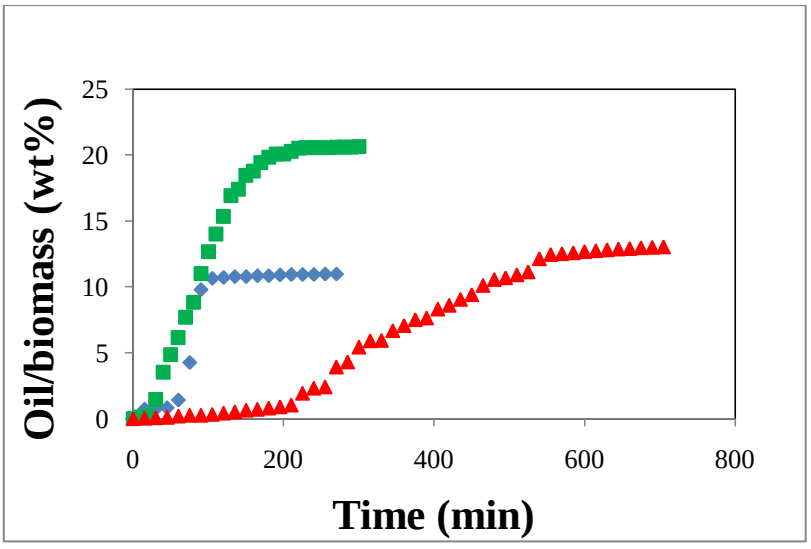


***Figure 3.12**. Effect of pressure and temperature at 1 g/min $CO_2$ flow rate. Blue:300 bar and 35°C, Red: 150 bar and 35°C, Green: 300 bar and 70°C.*

In Table 3.3 a summary of the previous results is represented.

***Table 3.3.*** *Influence of pressure and temperature on extraction parameters.*

| Pressure (bar) | Temperature (°C) | Yield (%wt.) | $CO_2$ density ($kg/m^3$) | Solubility (g oil/kg $CO_2$) | Extraction time (min) |
|---|---|---|---|---|---|
| 300 | 35 | 10.9±1 | 929 | 6.8 | 270 |
| 300 | 70 | 20.6±1 | 787 | 4.8 | 300 |
| 150 | 35 | 13±1 | 792 | 1.1 | 705 |

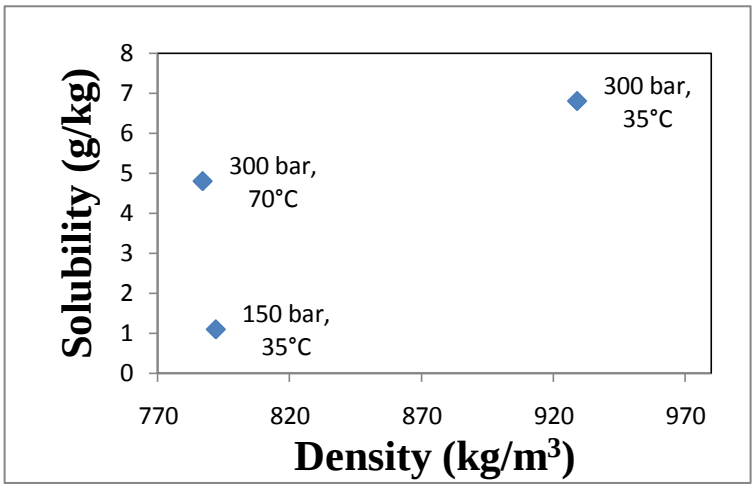


***Figure 3.13.*** *Oil solubility in SC-$CO_2$ in function of solvent density.*

A great difference in oil solubilities among the three conditions tested was obtained, as shown in Figure 3.13; in particular, the solubility at 150 bar and 35°C appears too low compared to that at 300 bar and 70°C, because the density of SC-$CO_2$ was almost the same (i.e. about 790 $kg/m^3$, Table 3.3) and solubility strongly depends on the solvent density. This difference could be attributed to the effect of the vapor pressure of the solute (i.e. Poynting effect); in fact, the volatility of a compound increases exponentially with increasing temperature and so, at constant density, solubility increases with temperature. An alternative motivation could be the incomplete recovery of the extracted oil in the assays at low pressure; no solvent was used to recover the oil after the BPR valve and it could be stuck in the pipe between the valve and the glass flask.

Finally, the best extraction yield was obtained at 300 bar, 70°C and 1 g/min, but the fastest extraction was at 300 bar, 35°C and 5 g/min. A good compromise between high extraction yields and short extraction times, on the other side, was achieved with 3 g/min

flow rate, similar to the results obtained by Viguera *et al.*. Further optimization of pressure and temperature conditions using the flow rate of 3 g/min is expected to improve the process.

# Conclusions

The aims of this project were to firstly develop a cheap system, compared to an automated bioreactor, in order to optimize heterotrophic growth conditions of a promising microalgae strain, *Chlorella protothecoides*. Secondly, various supercritical extraction assays were performed in this work in order to improve the results obtained in the previous work by Viguera *et al.* (2012), using the same biomass but varying type of extractor, its geometry and operative conditions.

Microalgae cultivation was firstly performed in batch mode in 250-ml Erlenmeyer flasks for 3 days at 28°C in order to analyze the most suitable among two media already used in literature with promising results and to evaluate the influence of dark/light conditions over the microalgal growth rate. Medium 1, which was taken from the study by Xiong *et al.* (2008), proved to be superior in comparison to medium 2 (Chen and Walker, 2011): the difference could be due to the inhibitory effect of the higher initial glucose concentration in medium 2. As regards the influence of light conditions, cultivation in dark resulted in a higher growth rate, almost double; dark conditions might have completely avoided photoautotrophy and ensured heterotrophic growth.
Medium 1 and dark conditions were chosen for the first scale-up to 1-l Erlenmeyer flasks, with pH control and fed-batch strategy. After 7 days of culture, biomass concentration was 21.6±0.5 g/l; comparing to batch culture in 250-ml flasks for 5 days (7.9±0.5 g/l), a similar slope was observed in the first 3 days and then, due to the consumption of the initial glucose content in the medium, growth in batch culture ended and quite constant biomass concentration was observed, whereas in fed-batch strategy microalgae presented a continuous growth rate with practically a constant slope. Moreover, a biomass productivity of 2.87 g/l/d was observed, much higher than in batch culture (1.58 g/l/d).
A further scale-up to 3.5 l of medium in a common aquarium provided of compressed air line was performed. The biomass concentration reached was of 44.5 g/l in 185 h of culture, lower than that obtained by Xiong *et al.* of 52.5 g/l in 168 h of culture, but similar to that obtained by Chen and Walker of 46 g/l. The slope of all the growth curves is quite similar; however, in this study the culture started from a lower initial biomass concentration and that could have been a discriminating factor. Moreover, the equipment used in the present study was much more cheaper, “rudimental”, though more difficult to

control than a bioreactor. In particular, the temperature control system will have to be more controlled and precise for further developments, as for example a smaller size aquarium compatible with the size of an incubator and with an internal thermocouple to measure temperature constantly. Farther, compressed air flow rate was not controlled with any automatic system, but nevertheless growth rate was very high. Finally, a first scale up of the process was achieved with good results. The developed system, surely cheaper compared to an automated bioreactor, was able to grow microalgae on a lab-scale with high and fast biomass productivity. A further scale-up to an industrial scale can also be aimed, because this system is very similar to a common wastewater biological oxidation tank, provided that contamination is avoided.

Supercritical extractions were performed with a 30 ml extractor instead of 100 ml, and the length to diameter ratio was changed from 1:3 to 8:1. Assays were performed at 35°C, 300 bar and 1 g $CO_2$/min. Temperature, instead of 40°C, was slightly lowered in order to reach a greater solubility of microalgae oil and for energy saving reasons.
Extractions performed in the previous work led to only 6.3%wt. oil extracted in contrast with 10.9±1%wt. Moreover, the extraction time was drastically reduced from 750 min to 270 min. Also, oil solubility in carbon dioxide was drastically improved and reached 6.8 g oil/kg $CO_2$, comparable to solubility data found in literature. Bed geometry was identified as a discriminating parameter, because of a better distribution of supercritical carbon dioxide in the fixed bed of the extractor due to a better particle diameter to extractor diameter ratio that reduced the formation of preferential channels and higher linear velocities that led to higher external mass transfer coefficients.
The increase in flow rate from 1 to 3 and 5 g/min led to a higher extraction yield and to shorter extraction times (even if the consume of $CO_2$ was larger), an increase in the linear velocity of the process, Re number and external mass transfer coefficient (which were in good agreement with other reported values). The flow rate of 3 g/min had the highest extraction yield but not the fastest extraction time, which was achieved with 5 g/min. Anyway, only with 1 g/min $CO_2$ flow rate the extraction yield was better than in the previous work; with higher flow rates results are very similar, although a little bit higher in the work by Viguera *et al.*. It is suggested that with the lowest flow rate used, $CO_2$ saturation with oil was better than with the higher flow rates. Moreover, a decrease in oil solubility was noticed as the flow rate increased in both studies, and this might be due to an insufficient saturation of $CO_2$ with oil or to channeling in the extractor.
A decrease in pressure led to lower oil solubility, longer extraction times and only a small increase in the quantity of extracted oil. An increase in temperature led to a lower solubility but also to a higher extraction yield, about two times, in practically the same time of extraction. The decrease in oil solubility was not so marked as in the assay at

lower pressure, but was unexpected because this would mean that the crossover pressure is at pressures lower than 300 bar.

Finally, a good compromise between high extraction yields and short extraction times was achieved with 3 g/min flow rate, similar to the results obtained by Viguera *et al.*. Further optimization of pressure and temperature conditions using this flow rate is expected to improve the process.

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